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(54) Title: MASS TAG PCR FOR MULTIPLEX DIAGNOSTICS

(57) Abstract: This invention provides a mass tag-based method for simultaneously detecting in a sample the presence of one or more of a plurality of different target nucleic acids. This invention also provides related kits.

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MASS TAG PCR FOR MULTIPLEX DIAGNOSTICS

5 This application claims priority of U.S. Provisional Application No. 60/566,967, filed April 29, 2004, the contents of which are hereby incorporated by reference.

10 The invention disclosed herein was made with Government support under grant no. AI51292 from the National Institutes of Health. Accordingly, the U.S. Government has certain rights in this invention.

15 Throughout this application, various publications are referenced. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe
20 the state of the art to which this invention pertains.

Background of the Invention

25 Establishing a causal relationship between infection with a virus and a specific disease may be complex. In most acute viral diseases, the responsible agent is readily implicated because it replicates at high levels in the affected tissue at the time the disease is manifest, morphological changes consistent with infection are
30 evident, and the agent is readily cultured with standard microbiological techniques. In contrast, implication of viruses in chronic diseases may be confounded because persistence requires restricted gene expression, classical hallmarks of infection are absent, and/or

mechanisms of pathogenesis are indirect or subtle.

Methods for cloning nucleic acids of microbial pathogens directly from clinical specimens offer new opportunities
5 to investigate microbial associations in chronic diseases. The power of these methods is that they can succeed where methods for pathogen identification through serology or cultivation may fail due to absence of specific reagents or fastidious requirements for agent
10 replication. Over the past decade, the application of molecular pathogen discovery methods resulted in identification of novel agents associated with both acute and chronic diseases, including Borna disease virus, Hepatitis C virus, Sin Nombre virus, HHV-6, HHV-8,
15 Bartonella henselae, and Tropheryma whippeli.

Various methods are employed or proposed for cultivation-independent characterization of infectious agents. These can be broadly segregated into methods based on direct
20 analysis of microbial nucleic acid sequences (e.g., cDNA microarrays, consensus PCR, representational difference analysis, differential display), direct analysis of microbial protein sequences (e.g., mass spectrometry), immunological systems for microbe detection (e.g.,
25 expression libraries, phage display) and host response profiling. A comprehensive program in pathogen discovery would need to exploit most, if not all, of these technologies.

30 The decision to employ a specific method is guided by the clinical features, epidemiology, and spectrum of potential pathogens to be implicated. Expression

libraries, comprised of cDNAs or synthetic peptides, may be useful tools in the event that large quantities of acute and convalescent sera or cerebrospinal fluid are available for screening purposes; however, the approach
5 is cumbersome, labor-intensive, and success is dependent on the presence of a specific, high affinity humoral immune response. The utility of host response mRNA profile analysis has been demonstrated in several *in vitro* paradigms and some inbred animal models;
10 nonetheless, it is important to formally consider the possibility that a variety of organisms may activate similar cascades of chemokines, cytokines, and other soluble factors that influence host gene expression to produce what are likely to be convergent gene expression
15 profiles. Thus, at least in virology, it is prudent to explore complementary methods for pathogen identification based on agent-encoded nucleic acid motifs. Given the potential for high density printing of microarrays, it is feasible to design slides or chips decorated with both
20 host and pathogen targets. This would provide an unprecedented opportunity to simultaneously survey host response mRNA profiles and viral flora, providing insights into microbial pathogenesis not apparent with either method of analysis alone.

25
Representational difference analysis (RDA) is an important tool for pathogen identification and discovery. However, RDA is a subtractive cloning method for binary comparisons of nucleic acid populations. Thus, although
30 ideal for analysis of cloned cells or tissue samples that differ only in a single variable of interest, RDA is less well suited to investigation of syndromes wherein

infection with any of several different pathogens results in similar clinical manifestations, or infection is not invariably associated with disease. An additional caveat is that because the method is dependent upon the presence
5 of a limited number of restriction sites, RDA is most likely to succeed for agents with large genomes. Indeed, in this context, it is noteworthy that the two viruses detected by RDA in the listing above were herpesviruses.

10 Consensus PCR (cPCR) has been a remarkably productive tool for biology. In addition to identifying pathogens, particularly genomes of prokaryotic pathogens, this method has facilitated identification of a wide variety of host molecules, including cytokines, ion channels, and
15 receptors. Nonetheless, until recently, a difficulty in applying cPCR to pathogen discovery in virology has been that it is difficult to identify conserved viral sequences of sufficient length to allow cross-hybridization, amplification, and discrimination using
20 traditional cPCR format. While this may not be problematic when one is targeting only a single virus family, the number of assays required becomes infeasible when preliminary data are insufficient to allow a directed, limited analysis.

25 Real-time PCR methods have significantly changed diagnostic molecular microbiology by providing rapid, sensitive, specific tools for detecting and quantitating genetic targets. Because closed systems are employed,
30 real-time PCR is less likely than nested PCR to be confounded by assay contamination due to inadvertent aerosol introduction of amplicon/positive control/cDNA

templates that can accumulate in diagnostic laboratories. The specificity of real time PCR is both a strength and a limitation. Although the potential for false positive signal is low so is the utility of the method for
5 screening to detect related but not identical genetic targets. Specificity in real-time PCR is provided by two primers (each approximately 20 matching nucleotides (nt) in length) combined with a specific reporter probe of about 27 nt. The constraints of achieving hybridization
10 at all three sites may confound detection of diverse, rapidly evolving microbial genomes such as those of single-stranded RNA viruses. These constraints can be compensated in part by increasing numbers of primer sets accommodating various templates. However, because real-
15 time PCR relies on fluorescent reporter dyes, the capacity for multiplexing is limited to the number of emission peaks that can be unequivocally separated. At present up to four dyes can be identified simultaneously. Although the repertoire may increase, it will not likely
20 change dramatically.

Summary of the Invention

This invention provides a method for simultaneously detecting in a sample the presence of one or more of a plurality of different target nucleic acids comprising the steps of:

- (a) contacting the sample with a plurality of nucleic acid primers simultaneously and under conditions permitting, and for a time sufficient for, primer extension to occur, wherein (i) for each target nucleic acid at least one predetermined primer is used which is specific for that target nucleic acid, (ii) each primer has a mass tag of predetermined size bound thereto via a labile bond, and (iii) the mass tag bound to any primer specific for one target nucleic acid has a different mass than the mass tag bound to any primer specific for any other target nucleic acid;
 - (b) separating any unextended primers from any extended primers;
 - (c) simultaneously cleaving the mass tags from any extended primers; and
 - (d) simultaneously determining the presence and sizes of any mass tags so cleaved,
- wherein the presence of a cleaved mass tag having the same size as a mass tag of predetermined size previously bound to a predetermined primer indicates the presence in the sample of the target nucleic acid specifically recognized by that predetermined primer.

30

This invention further provides the instant method, wherein the method detects the presence in the sample of

10 or more, 50 or more, 100 or more, or 200 or more
different target nucleic acids. This invention further
provides the instant method, wherein the sample is
contacted with 4 or more, or 10 or more, or 50 or more,
5 or 100 or more, or 200 or more different primers.

This invention further provides the instant method,
wherein one or more primers comprises the sequence set
forth in one of SEQ ID NOs:1-96, and 98-101. This
10 invention further provides the instant method, wherein at
least two different primers are specific for the same
target nucleic acid. This invention further provides the
instant method, wherein a first primer is a forward
primer for the target nucleic acid and a second primer is
15 a reverse primer for the same target nucleic acid.

This invention further provides the instant method,
wherein the mass tags bound to the first and second
primers are of the same size. This invention further
20 provides the instant method, wherein the mass tags bound
to the first and second primers are of a different size.

This invention further provides the instant method,
wherein at least one target nucleic acid is from a
25 pathogen.

This invention further provides the instant method,
wherein the presence and size of any cleaved mass tag is
determined by mass spectrometry. This invention further
30 provides the instant method, wherein the mass
spectrometry is selected from the group consisting of
atmospheric pressure chemical ionization mass

spectrometry, electrospray ionization mass spectrometry,
and matrix assisted laser desorption ionization mass
spectrometry.

Brief Description of the Figures

Figure 1: This figure shows the structure of mass tag precursors and four photoactive mass tags.

5

Figure 2: This figure shows an ACPI mass spectrum of mass tag precursors for digital virus detection.

Figure 3: This figure shows DNA sequencing sample preparation for MS analysis using biotinylated dideoxynucleotides and a streptavidin coated solid phase.

10

Figure 4: This figure shows a mass spectrum from Sanger sequencing reactions using dd(A, G, C)TP-11-biotin and ddTTP-16-biotin.

15

Figure 5: This figure shows synthesis of NHS ester of one mass tag for tagging amino-primer (SEQ ID NO:97).

Figure 6: This figure shows the general structure of mass tags and photocleavage mechanism to release the mass tags from DNA for MS detection.

20

Figure 7: This figure shows four mass tagged biotinylated ddNTPs.

25

Figure 8: This figure shows the structure of four mass tag precursors and the four photoactive mass tags.

Figure 9: This figure shows APCI mass spectra for four mass tags after cleavage from primers. 2-nitrosacetophenone, m/z 150; 4 fluoro-2-

30

nitrosacetophenone, m/z 168; 5-methoxy-2-nitrosacetophenone, m/z 180; and 4,5-dimethoxy-2-nitrosacetophenone.

5 Figure 10: This figure shows four mass tag-labeled DNA molecules.

Figure 11: This figure shows differential real-time PCR for HCoV SARS, OC43, and 229E.

10

Figure 12: This figure shows 58 tags cleaved from oligonucleotides and detected using ACPI-MS. Each peak represents a different tag structure as a unique signature of the oligonucleotide it was originally
15 attached to.

Figure 13: This figure shows singleplex mass tag PCR for (1) influenza A virus matrix protein, (2) human coronavirus SARS, (3) 229E, (4) OC43, and (5) the
20 bacterial agent M. pneumoniae. (6) shows a 100bp ladder.

Figure 14: This figure shows mass spectrum representative of data collected using a miniaturized cylindrical ion trap mass analyzer coupled with a corona discharge
25 ionization source.

Figure 15: This figure shows mass spectrum of perfluoro-dimethylcyclohexane collected on a prototype atmospheric sampling glow discharge ionization source.

30

Figure 16: This figure shows the sensitivity of a 21-plex mass tag PCR. Dilutions of cloned gene target standards

(10 000, 1 000, 500, 100 molecules/assay) diluted in human placenta DNA were analyzed by mass tag PCR. Each reaction mix contained 2x Multiplex PCR Master Mix (Qiagen), the indicated standard and 42 primers at 1X nM concentration labeled with different mass tags. Background in reactions without standard (no template control, 12.5 ng human DNA) was subtracted and the sum of Integrated Ion Current for both tags was plotted.

10 Figure 17: This figure shows analysis of clinical specimens; respiratory infection. RNA from clinical specimens was extracted by standard procedures and reverse transcribed into cDNA (Superscript RT system, Invitrogen, Carlsbad, CA; 20 ul volume). Five microliter
15 of reaction was then subjected to mass tag PCR.

Figure 18: This figure shows multiplex mass tag PCR analysis of six human respiratory specimens. Mass tag primer sets employed in a single tube assay are indicated
20 at the bottom of the figure.

Figure 19: This figure shows structures of MASSCODE tags.

Figure 20: This figure shows differential real-time PCR
25 for West Nile virus and St. Louis encephalitis virus.

Figures 21A-21B: (A) This figure shows serial dilutions of plasmid standards (5×10^5 , 5×10^4 , 5×10^3 , 5×10^2 , 5×10^1 , and 5×10^0) for RSV group A, RSV group B, Influenza A, HCoV-SARS, HCoV-229E, HCoV-OC43, and M. pneumoniae were each analyzed by mass tag PCR in a
30 multiplex format. (B) This figure shows simultaneous

detection of multiple targets in multiplex format using mixtures of two templates per assay (5×10^4 copies each): HCoV-SARS and M. pneumoniae, HCoV-229E and M. pneumoniae, HCoV-OC43 and M. pneumoniae, and HCoV-229E and HCoV-OC43.

5

Figure 22: This figure shows a schematic of the mass tag PCR procedure.

Figure 23: Thus figure shows identification of various
10 infections using masscode tags.

Detailed Description of the Invention

Terms

5

As used herein, and unless stated otherwise, each of the following terms shall have the definition set forth below.

10 "Mass tag" shall mean any chemical moiety (i) having a fixed mass, (ii) affixable to a nucleic acid, and (iii) whose mass is determinable using mass spectrometry. Mass tags include, for example, chemical moieties such as small organic molecules, and have masses which range, for
15 example, from 100Da to 2500Da.

"Nucleic acid" shall mean any nucleic acid molecule, including, without limitation, DNA, RNA and hybrids thereof. The nucleic acid bases that form nucleic acid
20 molecules can be the bases A, C, G, T and U, as well as derivatives thereof. Derivatives of these bases are well known in the art, and are exemplified in PCR Systems, Reagents and Consumables (Perkin Elmer Catalogue 1996-1997, Roche Molecular Systems, Inc., Branchburg, New
25 Jersey, USA).

"Pathogen" shall mean an organic entity including, without limitation, viruses and bacteria, known or suspected to be involved in the pathogenesis of a disease
30 state in an organism such as an animal or human.

"Sample" shall include, without limitation, a biological

sample derived from an animal or a human, such as cerebro-spinal fluid, lymph, blood, blood derivatives (e.g. sera), liquidized tissue, urine and fecal material.

5 "Simultaneously detecting", with respect to the presence of target nucleic acids in a sample, means determining, in the same reaction vessels(s), whether none, some or all target nucleic acids are present in the sample. For example, in the instant method of simultaneously
10 detecting in a sample the presence of one or more of 50 target nucleic acids, the presence of each of the 50 target nucleic acids will be determined simultaneously, so that results of such detection could be, for example, (i) none of the target nucleic acids are present, (ii)
15 five of the target nucleic acids are present, or (iii) all 50 of the target nucleic acids are present.

"Specific", when used to describe a primer in relation to a target nucleic acid, shall mean that, under primer
20 extension-permitting conditions, the primer specifically binds to a portion of the target nucleic acid and is extended.

"Target nucleic acid" shall mean a nucleic acid whose
25 presence in a sample is to be detected by any of the instant methods.

"5-UTR" shall mean the 5'-end untranslated region of a nucleic that encodes a protein.

30

The following abbreviations shall have the meanings set forth below: "A" shall mean Adenine; "bp" shall mean base

pairs; "C" shall mean Cytosine; "DNA" shall mean deoxyribonucleic acid; "G" shall mean Guanine; "mRNA" shall mean messenger ribonucleic acid; "RNA" shall mean ribonucleic acid; "PCR" shall mean polymerase chain
5 reaction; "T" shall mean Thymine; "U" shall mean Uracil; "Da" shall mean dalton.

Finally, with regard to the embodiments of this invention, where a numerical range is stated, the range
10 is understood to encompass the embodiments of each and every integer between the lower and upper numerical limits. For example, the numerical range from 1 to 5 is understood to include 1, 2, 3, 4, and 5.

15 Embodiments of the Invention

To address the need for enhanced multiplex capacity in diagnostic molecular microbiology we have established a PCR platform based on mass tag reporters that are easily
20 distinguished in Mass Spectrometry (MS) as discrete signal peaks. Major advantages of the PCR/MS system include: (1) hybridization to only two sites is required (forward and reverse primer binding sites) vs real time PCR where an intermediate third oligonucleotide is used
25 (probe binding site); this enhances flexibility in primer design; (2) tried and proven consensus PCR primers can be adapted to PCR/MS; this reduces the time and resources that must be invested to create new reagents and assay controls; (3) the large repertoire of tags allows highly
30 multiplexed assays; additional tags can be easily synthesized to allow further complexity; and (4) sensitivity of real time PCR is maintained. We view

PCR/MS as a tool with which to rapidly screen clinical materials for the presence of candidate pathogens. Thereafter, targeted secondary tests, including real time PCR, can be used to quantitate microbe burden and pursue
5 epidemiologic studies.

Specifically, this invention provides a method for simultaneously detecting in a sample the presence of one or more of a plurality of different target nucleic
10 acids comprising the steps of:

- (a) contacting the sample with a plurality of nucleic acid primers simultaneously and under conditions permitting, and for a time sufficient for, primer extension to occur, wherein (i) for each target
15 nucleic acid at least one predetermined primer is used which is specific for that target nucleic acid, (ii) each primer has a mass tag of predetermined size bound thereto via a labile bond, and (iii) the mass tag bound to any primer specific
20 for one target nucleic acid has a different mass than the mass tag bound to any primer specific for any other target nucleic acid;
- (b) separating any unextended primers from any extended primers;
- 25 (c) simultaneously cleaving the mass tags from any extended primers; and
- (d) simultaneously determining the presence and sizes of any mass tags so cleaved,

wherein the presence of a cleaved mass tag having the
30 same size as a mass tag of predetermined size previously bound to a predetermined primer indicates the presence in the sample of the target nucleic acid specifically

recognized by that predetermined primer.

In one embodiment of the instant method, the method detects the presence in the sample of 10 or more
5 different target nucleic acids. In another embodiment, the method detects the presence in the sample of 50 or more different target nucleic acids. In a further embodiment, the method detects the presence in the sample of 100 or more different target nucleic acids. In a
10 further embodiment, the method detects the presence in the sample of 200 or more different target nucleic acids.

In one embodiment of the instant method, the sample is contacted with 4 or more different primers. In another
15 embodiment, the sample is contacted with 10 or more different primers. In a further embodiment, the sample is contacted with 50 or more different primers. In a further embodiment, the sample is contacted with 100 or more different primers. In yet a further embodiment, the
20 sample is contacted with 200 or more different primers.

In one embodiment of the instant method, one or more primers comprises the sequence set forth in one of SEQ ID
25 NOS:1-96, and 98-101.

In another embodiment of the instant method, at least two different primers are specific for the same target nucleic acid. For example, in one embodiment a first primer is a forward primer for the target nucleic acid
30 and a second primer is a reverse primer for the same target nucleic acid. In this example, the mass tags bound to the first and second primers can be of the same size

or of different sizes. In another embodiment, a first primer is directed to a 5'-UTR of the target nucleic acid and a second primer is directed to a 3D polymerase region of the target nucleic acid.

5

In one embodiment of the instant method, wherein each primer is from 15 to 30 nucleotides in length. In another embodiment, each mass tag has a molecular weight of from 100Da to 2,500Da. In a further embodiment, the labile
10 bond is a photolabile bond, such as a photolabile bond cleavable by ultraviolet light.

In another embodiment of the instant method, at least one target nucleic acid is from a pathogen. Pathogens
15 include, without limitation, B. anthracis, a Dengue virus, a West Nile virus, Japanese encephalitis virus, St. Louis encephalitis virus, Yellow Fever virus, La Crosse virus, California encephalitis virus, Rift Valley Fever virus, CCHF virus, VEE virus, EEE virus, WEE virus,
20 Ebola virus, Marburg virus, LCMV, Junin virus, Machupo virus, Variola virus, SARS corona virus, an enterovirus, an influenza virus, a parainfluenza virus, a respiratory syncytial virus, a bunyavirus, a flavivirus, and an alphavirus.

25

In another embodiment, the pathogen is a respiratory pathogen. Respiratory pathogens include, for example, respiratory syncytial virus A, respiratory syncytial virus B, Influenza A (N1), Influenza A (N2), Influenza A
30 (M), Influenza A (H1), Influenza A (H2), Influenza A (H3), Influenza A (H5), Influenza B, SARS coronavirus, 229E coronavirus, OC43 coronavirus, Metapneumovirus

European, Metapneumovirus Canadian, Parainfluenza 1,
Parainfluenza 2, Parainfluenza 3, Parainfluenza 4A,
Parainfluenza 4B, Cytomegalovirus, Measles virus,
Adenovirus, Enterovirus, M. pneumoniae, L. pneumophila,
5 and C. pneumoniae.

In a further embodiment, the pathogen is an encephalitis-
inducing pathogen. Encephalitis-inducing pathogens
include, for example, West Nile virus, St. Louis
10 encephalitis virus, Herpes Simplex virus, HIV 1, HIV 2,
N. meningitides, S. pneumoniae, H. influenzae, Influenza
B, SARS coronavirus, 229E-CoV, OC43-CoV, Cytomegalovirus,
and a Varicella Zoster virus. In a further embodiment,
the pathogen is a hemorrhagic fever-inducing pathogen. In
15 a further embodiment, the sample is a forensic sample, a
food sample, blood, or a derivative of blood, a
biological warfare agent or a suspected biological
warfare agent.

20 In one embodiment of the instant method, the mass tag is
selected from the group consisting of structures V1 to V4
of Fig. 1 or Fig. 8.

In another embodiment of the instant method, the presence
25 and size of any cleaved mass tag is determined by mass
spectrometry. Mass spectrometry includes, for example,
atmospheric pressure chemical ionization mass
spectrometry, electrospray ionization mass spectrometry,
and matrix assisted laser desorption ionization mass
30 spectrometry.

In one embodiment of the instant method, the target

nucleic acid is a ribonucleic acid. In another embodiment, the target nucleic acid is a deoxyribonucleic acid. In a further embodiment, the target nucleic acid is from a viral source.

5

This invention provides a kit for simultaneously detecting in a sample the presence of one or more of a plurality of different target nucleic acids comprising a plurality of nucleic acid primers wherein (i) for each
10 target nucleic acid at least one predetermined primer is used which is specific for that target nucleic acid, (ii) each primer has a mass tag of predetermined size bound thereto via a labile bond, and (iii) the mass tag bound to any primer specific for one target nucleic acid has a
15 different mass than the mass tag bound to any primer specific for any other target nucleic acid.

This invention also provides a kit for simultaneously detecting in a sample the presence of one or more of a
20 plurality of different target nucleic acids comprising (a) a plurality of nucleic acid primers wherein (i) for each target nucleic acid at least one predetermined primer is used which is specific for that target nucleic acid, (ii) each primer has a mass tag of predetermined
25 size bound thereto via a labile bond, and (iii) the mass tag bound to any primer specific for one target nucleic acid has a different mass than the mass tag bound to any primer specific for any other target nucleic acid; and (b) a mass spectrometer.

30

This invention further provides a kit for simultaneously detecting in a sample the presence of one or more of a

plurality of different target nucleic acids comprising
(a) a plurality of nucleic acid primers wherein (i) for
each target nucleic acid at least one predetermined
primer is used which is specific for that target nucleic
5 acid, (ii) each primer has a mass tag of predetermined
size bound thereto via a labile bond, and (iii) the mass
tag bound to any primer specific for one target nucleic
acid has a different mass than the mass tag bound to any
primer specific for any other target nucleic acid, and
10 (b) instructions for use.

Finally, this invention provides a kit for simultaneously
detecting in a sample the presence of one or more of a
plurality of different target nucleic acids comprising
15 (a) a plurality of nucleic acid primers wherein (i) for
each target nucleic acid at least one predetermined
primer is used which is specific for that target nucleic
acid, (ii) each primer has a mass tag of predetermined
size bound thereto via a labile bond, and (iii) the mass
20 tag bound to any primer specific for one target nucleic
acid has a different mass than the mass tag bound to any
primer specific for any other target nucleic acid; (b) a
mass spectrometer; and (c) instructions for
simultaneously detecting in a sample the presence of one
25 or more of a plurality of different target nucleic acids
using the primers and the mass spectrometer.

This invention will be better understood by reference to
the Experimental Details which follow, but those skilled
30 in the art will readily appreciate that the specific
experiments detailed are only illustrative of the
invention as described more fully in the claims which

follow thereafter.

Experimental Details

5

Example 1

Abbreviations: 5'-UTR, 5'-untranslated region; ALS, Amyotrophic Lateral Sclerosis; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; PCR, polymerase chain reaction; MALDI-TOF, matrix assisted laser desorption ionization time of flight; MS, mass spectrometry

Background

15

Establishing a causal relationship between infection with a virus and a specific disease may be complex. In most acute viral diseases, the responsible agent is readily implicated because it replicates at high levels in the affected tissue at the time the disease is manifest, morphological changes consistent with infection are evident, and the agent is readily cultured with standard microbiological techniques. In contrast, implication of viruses in chronic diseases may be confounded because persistence requires restricted gene expression, classical hallmarks of infection are absent, and/or mechanisms of pathogenesis are indirect or subtle. Methods for cloning nucleic acids of microbial pathogens directly from clinical specimens offer new opportunities to investigate microbial associations in chronic diseases (21). The power of these methods is that they can succeed where methods for pathogen identification through

serology or cultivation may fail due to absence of specific reagents or fastidious requirements for agent replication. Over the past decade, the application of molecular pathogen discovery methods resulted in
5 identification of novel agents associated with both acute and chronic diseases, including Borna disease virus, Hepatitis C virus, Sin Nombre virus, HHV-6, HHV-8, *Bartonella henselae*, and *Tropherema whippeli* (5-7, 17, 19, 22, 23, 27).

10

Various methods are employed or proposed for cultivation-independent characterization of infectious agents. These can be broadly segregated into methods based on direct analysis of microbial nucleic acid sequences (e.g., cDNA
15 microarrays, consensus PCR, representational difference analysis, differential display), direct analysis of microbial protein sequences (e.g., mass spectrometry), immunological systems for microbe detection (e.g., expression libraries, phage display) and host response
20 profiling. A comprehensive program in pathogen discovery will need to exploit most, if not all, of these technologies.

The decision to employ a specific method is guided by the
25 clinical features, epidemiology, and spectrum of potential pathogens to be implicated. Expression libraries, comprised of cDNAs or synthetic peptides, may be useful tools in the event that large quantities of acute and convalescent sera or cerebrospinal fluid are
30 available for screening purposes; however, the approach is cumbersome, labor-intensive, and success is dependent on the presence of a specific, high affinity humoral

immune response. The utility of host response mRNA profile analysis has been demonstrated in several in vitro paradigms and some inbred animal models (8, 26, 30); nonetheless, it is important to formally consider

5 the possibility that a variety of organisms may activate similar cascades of chemokines, cytokines, and other soluble factors that influence host gene expression to produce what are likely to be convergent gene expression profiles. Thus, at least in virology, it is prudent to

10 explore complementary methods for pathogen identification based on agent-encoded nucleic acid motifs. Given the potential for high density printing of microarrays, it is feasible to design slides or chips decorated with both host and pathogen targets. This would provide an

15 unprecedented opportunity to simultaneously survey host response mRNA profiles and viral flora, providing insights into microbial pathogenesis not apparent with either method of analysis alone. Representational difference analysis (RDA) is an important tool for

20 pathogen identification and discovery. However, RDA is a subtractive cloning method for binary comparisons of nucleic acid populations (12, 18). Thus, although ideal for analysis of cloned cells or tissue samples that differ only in a single variable of interest, RDA is less

25 well suited to investigation of syndromes wherein infection with any of several different pathogens results in similar clinical manifestations, or infection is not invariably associated with disease. An additional caveat is that because the method is dependent upon the presence

30 of a limited number of restriction sites, RDA is most likely to succeed for agents with large genomes. Indeed, in this context, it is noteworthy that the two viruses

detected by RDA in the listing above (see first paragraph) were herpesviruses (5, 6). Consensus PCR (cPCR) has been a remarkably productive tool for biology. In addition to identifying pathogens, particularly
5 genomes of prokaryotic pathogens, this method has facilitated identification of a wide variety of host molecules, including cytokines, ion channels, and receptors. Nonetheless, until recently, a difficulty in applying cPCR to pathogen discovery in virology has been
10 that it is difficult to identify conserved viral sequences of sufficient length to allow cross-hybridization, amplification, and discrimination using traditional cPCR format. While this may not be problematic when one is targeting only a single virus
15 family, the number of assays required becomes infeasible when preliminary data are insufficient to allow a directed, limited analysis. To address this issue, we adapted cPCR to Differential Display, a PCR-based method for simultaneously displaying the genetic composition of
20 multiple sample populations in an acrylamide gel format (16). This hybrid method, domain-specific differential display (DSDD), employs short, degenerate primer sets designed to hybridize to viral genes representing larger taxonomic categories than can be resolved in cPCR. The
25 major advantages to this approach are: (i) reduction in numbers of reactions required to identify genomes of known viruses, and (ii) potential to detect viruses less closely related to known viruses than those found through cPCR. The differential display format also permits
30 identification of syndrome-specific patterns of gene expression (host and pathogen) that need not be present in all clinical samples. Additionally, because multiple

samples can be analyzed in side-by-side comparisons, DSDD allows examination of the timecourse of gene expression patterns. Lastly, recent experience with isolation of the West Nile virus responsible for the outbreak of encephalitis in New York in the summer of 1999 indicates that DSDD may be advantageous in instances where template is suboptimal due to degradation (e.g., postmortem field specimens).

The development and application of sensitive high throughput methods for detecting a wide range of viruses is anticipated to provide new insights into the pathogenesis of chronic diseases. We are funded through AI51292 to support these objectives by establishing DNA microarray, multiplexed bead-based flow cytometric (MB-BFC) and domain specific differential display (DSDD) assay platforms for viral surveillance and discovery in chronic diseases. Each of these methods has its strengths; however, none is ideal. Microarrays provide a platform wherein one can simultaneously query thousands of microbial and host gene targets but lack sensitivity and are difficult to modify as new targets are identified. Bead-based arrays are flexible but similar in sensitivity to microarrays.

Domain specific differential display is sensitive and flexible but labor intensive. Real time PCR (not a component of our original application but useful to note for purposes of method comparisons), is rapid and sensitive, but cannot be used for broad range detection of viral sequences, because of stringent sequence constraints for the three oligonucleotides comprising the

system (two primers, one probe).

Mass-Tag PCR would integrate PCR and mass spectrometry (MS) into a stable and sensitive digital assay platform. It is similar in sensitivity and efficiency to real time PCR but provides the advantages of simultaneous detection and discrimination of multiple targets, due to less stringent constraints on primer selection. Additionally, whereas multiplexing is limited in real time PCR by overlapping fluorescence emission spectra, Mass-Tag PCR allows discrimination of a large repertoire of mass tags with molecular weights between 150 and 2500 daltons.

In Mass-Tag PCR, virus identity is be defined by the presence of label of a specific molecular weight associated with an amplification product. Primers are be designed such that the tag can be cleaved by irradiation with UV light. Following PCR, the amplification product can be immobilized on a solid support and excess soluble primer removed. After cleavage by UV irradiation (~350 nm), the released tag will be analyzed by mass spectrometry. Detection is sensitive, fast, independent of DNA fragment length, and ideally suited to the multiplex format required to survey clinical materials for infection with a wide range of infectious agents.

Results

Mass spectrometry (MS) is a rapid, sensitive method for detection of small molecules. With the development of new ionization techniques such as matrix assisted laser desorption ionization (MALDI) and electrospray ionization

(ESI), mass spectrometry has become an indispensable tool in many areas of biomedical research. Although these ionization methods are suitable for the analysis of bioorganic molecules, such as peptides and proteins, improvements in both detection and sample preparation will be required before mass spectrometry can be used to directly detect long DNA fragments. A major confound in exploiting MS for genetic investigation has been that long DNA molecules are fragmented during the analytic process. The mass tag approach overcomes this limitation by detecting small stable mass tags that serve as signatures for specific DNA sequences rather than the DNA sequences themselves.

Atmospheric pressure chemical ionization (APCI) has advantages over ESI and MALDI for some applications. Because buffer and inorganic salts impact ionization efficiency, performance in ESI is critically dependent upon sample preparation conditions. In MALDI, matrix must be added prior to sample introduction into the mass spectrometer; speed is often limited by the need to search for an ideal irradiation spot to obtain interpretable mass spectra. APCI requires neither desalting nor mixing with matrix to prepare crystals on a target plate. Therefore in APCI, mass tag solutions can be injected directly. Because mass tags are volatile and have small mass values, they are easily detected by APCI ionization with high sensitivity. The APCI mass tag system is easily scaled up for high throughput operation.

We have established methods for synthesis and APCI analysis of mass tags coupled to DNA fragments.

Precursors of four mass tags [(a) acetophenone; (b) 3-fluoroacetophenone; (c) 3,4-difluoroacetophenone; and (d) 3,4-dimethoxyacetophenone] are shown in Fig. 1. Upon nitration and reduction, the photoactive tags are produced and used to code for the identity of up to four different primer pairs (or target sequences). In a simulation experiment, we have obtained clean APCI mass spectra for the 4 mass tag precursors (a, b, c, d) as shown in Fig. 2. The peak with m/z of 121 is a, 139 is b, 157 is c and 181 is d. This result indicates that the 4 compounds we designed as mass tags are stable and produce discrete high resolution digital data in an APCI mass spectrometer. In the research described below, each of the unique m/z from each mass tag translates to the identity of a viral sequence (V) [Tag-1 ($m/z, 150$) = V-1; Tag-2 ($m/z, 168$) = V-2; Tag-3 ($m/z, 186$) = V-3; Tag-4 ($m/z, 210$) = V-4]. A variety of functional groups can be introduced to the mass tag parent structure for generating a large number of mass tags with different molecular weights. Thus, a library of primers labeled with mass tags that can discriminate between hundreds of viral sequence targets.

DNA sequencing with biotinylated dideoxynucleotides on a mass spectrometer

PCR amplification can be nonspecific; thus, products are commonly sequenced to verify their identity as bona fide targets. Here we apply the rapidity and sensitivity of mass tag analyses to direct MS-sequencing of PCR amplified transcripts.

MALDI-TOF MS has recently been explored widely for DNA sequencing. The Sanger dideoxy procedure (25) is used to generate the DNA sequencing fragments. The mass resolution in theory can be as good as one dalton; however, in order to obtain accurate measurement of the mass of the sequencing DNA fragments, the samples must be free from alkaline and alkaline earth salts and falsely stopped DNA fragments (fragments terminated at dNTPs instead of ddNTPs). Our method for preparing DNA sequencing fragments using biotinylated dideoxynucleotides and a streptavidin-coated solid phase is shown in Fig. 3. DNA template, dNTPs (A, C, G, T) and ddNTP-biotin (A-b, C-b, G-b, T-b), primer and DNA polymerase are combined in one tube. After polymerase extension and termination reactions, a series of DNA fragments with different lengths are generated. The sequencing reaction mixture is then incubated for a few minutes with a streptavidin-coated solid phase. Only the DNA sequencing fragments that are terminated with biotinylated dideoxynucleotides at the 3' end are captured on the solid phase. Excess primers, falsely terminated DNA fragments, enzymes and all other components from the sequencing reaction are washed away. The biotinylated DNA sequencing fragments are then cleaved off the solid phase by disrupting the interaction between biotin and streptavidin using ammonium hydroxide or formamide to obtain a pure set of DNA sequencing fragments. These fragments are then mixed with matrix (3-hydroxypicolinic acid) and loaded onto a mass spectrometer to produce accurate mass spectra of the DNA sequencing fragments. Since each type of nucleotide has a unique molecular mass, the mass difference between

adjacent peaks of the mass spectra gives the sequence identity of the nucleotides. In DNA sequencing with mass spectrometry, the purity of the samples directly affects the quality of the obtained spectra. Excess primers, salts, and fragments that are prematurely terminated in the sequencing reactions (false stops) will create extra noise and extraneous peaks (11). Excess primers can also dimerize to form high molecular weight species that give a false signal in mass spectrometry (29). False stops occur in DNA sequencing reaction when a deoxynucleotide rather than a dideoxynucleotide terminates a sequencing fragment. A deoxynucleotide terminated false stop has a mass difference of 16 daltons compared with its dideoxy counterpart. This mass difference is identical to the difference between adenine and guanine. Thus, false stops can be misinterpreted or interfere with existing peaks in the mass spectra. Our method is designed to eliminate these confounds. We previously established a procedure for accurately sequencing DNA using fluorescent dye-labeled primers and biotinylated dideoxynucleotides. In this procedure, accurate and clean DNA sequencing data were obtained by removing falsely stopped fragments prior to analysis through use of an intermediate purification step on streptavidin-coated magnetic beads (13, 14).

Sequencing experiments for a 55 bp synthetic template using MALDI-TOF mass spectrometry were recently performed (9). Four commercially available biotinylated dideoxynucleotides ddATP-11-biotin, ddGTP-11-biotin, ddCTP-11-biotin and ddTTP-11-biotin (NEN, Boston) were used to produce the sequencing ladder in a single tube by cycle sequencing. Clean sequence peaks were obtained on

the mass spectra, with the first peak being primer extended by one biotinylated dideoxynucleotide. Although the identity of A and G residues were determined unambiguously, C and T could not be differentiated because the one dalton mass difference between the ddCTP-11-biotin and ddTTP-11-biotin cannot be consistently resolved by using the current mass detector for DNA fragments. Nonetheless, these results confirmed that clean sequencing ladders can be obtained by capture/release of DNA sequencing fragments with biotin located on the 3' dideoxy terminators. The procedure has been improved by using biotinylated ddTTPs that have large mass differences in comparison to ddCTP-11-biotin. Pairing ddTTP-16-biotin (Enzo, Boston), which has a large mass difference in comparison to ddCTP-11-biotin, with ddATP-11-biotin, ddCTP-11-biotin, and ddGTP-11-biotin, allowed unambiguous sequence determination in the mass spectra (Fig. 4). Mass spectrum from Sanger sequencing reactions using dd(A,G,C)TP-11-biotin and ddTTP-16-biotin. All four bases are unambiguously identified in the spectrum. Data presented here were generated using a synthetic template mimicking a portion of the HIV type 1 protease gene. DNA sequencing was performed in one tube by combining the biotinylated ddNTPs, regular dNTPs, DNA polymerase, and reaction buffer (9).

Table 1

Cloned enterovirus targets		
Virus	5' UTR	pol
Echovirus 3	+	+
Echovirus 6	+	+
Echovirus 9	+	+
Echovirus 16	+	+
Echovirus 17	+	+
Echovirus 25	+	+
Echovirus 30	+	+
Poliovirus 1	+	+
Poliovirus 2	+	+
Poliovirus 3	+	+
Coxsackie A9	+	+
Coxsackie B2	+	+
In Propagation		
Coxsackie (A9), Coxsackie A16, Coxsackie B1, Coxsackie B3, Coxsackie B4, Coxsackie B5, Coxsackie B6, Echovirus 7, Echovirus 13, Echovirus 18		

Cloning viral targets as controls for Mass-Tag PCR

Multiple sequence alignment algorithms have been used by
5 our bioinformatics core to extract the most conserved
genomic regions amongst the GenBank published enteroviral
sequences. Regions wherein sequence conservation meets or
exceeds 80% for an enteroviral serogroup or genetically
related subgroup have been identified in the 5'-
10 untranslated region (UTR) and the polymerase gene (3D) of
the enterovirus genus. A representative collection of
virus isolates has been obtained to generate calibrated
standards for Mass-Tag PCR (Table 1). The current panel
includes 22 isolates representing all characterized
15 serogroups of pathogenic relevance (A, B, C, and D;
covering about 90% of all US enterovirus isolates in the
past 10 years; the remaining 10% include non-typed
isolates). Twelve isolates have been grown and the
relevant regions cloned for spotting onto DNA microarrays
20 and use as transcript controls for DSDD, multiplex bead
based, and real time PCR assays. Viruses can be
propagated in the appropriate cell lines to generate
working and library stocks (Rd, Vero, HeLa, Fibroblast,
or WI-38 cells). Library stocks can be frozen and
25 maintained in curated collections at -70°C. Viral RNA
can be extracted from working stocks using Tri-Reagent
(Molecular Research Center, Inc.). Purified RNA can be
reverse transcribed into cDNA using random hexamer
priming [to avoid 3' bias] (Superscript II,
30 Invitrogen/Life Technologies).

Target regions of 100-200 bp representing the identified

core sequences will be amplified by PCR from cDNA template using virus-specific primers. Products are cloned (via a single deoxyadenosine residue added in template-independent fashion by common Taq-polymerases to 3'-ends of amplification products) into the transcription vector pGEM T-Easy (Promega Corp.). After transformation and amplification in *Escherichia coli*, plasmids are analyzed by restriction mapping and automated dideoxy sequencing (Columbia Genome Center) to determine insert orientation and fidelity of PCR. Plasmid libraries will be maintained as both cDNAs and glycerol stocks.

Multiple sequence alignment algorithms can be used to identify highly conserved (>95%) sequence stretches of 20-30 bp length within the identified core sequences to serve as targets for primer design.

Synthesis of Primers for Use in Mass-Tag PCR

Highly conserved target regions within the core sequences suitable for primer design are identified by using multiple sequence alignment algorithms adjusted for the appropriate window size (20-30 bp) and conservation threshold (>95%). Final alignments are color-coded to facilitate manual inspection. Parameters implicated in primer performance including melting temperature, 3'-terminal stability, internal stability, and propensity of potential primers to form stem loops or primer-dimers can be assessed using standard primer selection software programs OLIGO (Molecular Biology Insights), Primer Express (PE Applied Biosystems), and Primer Premiere (Premiere Biosoft International). Primers can be

synthesized with a primary amine-group at the 5'-end for subsequent coupling to NHS esters of the mass tags (Fig. 5). Mass tags with molecular weights between 150 and 2500 daltons can be generated by introducing various functional groups [Rn] in the mass tag parent structure to code for individual primers and thus for the targeted viral sequence (see Fig. 6; also showing the photocleavage reaction). MS is capable of detecting small stable molecules with high sensitivity, a mass resolution greater than one dalton, and the detection requires only microseconds. The mass tagging approach has been successfully used to detect multiplex single nucleotide polymorphisms (15).

15 *Sensitivity and Specificity of Mass-Tag PCR for Detection of Enteroviral Transcripts*

Although the method disclosed here is useful for detecting viral RNA, plasmid DNA is an inexpensive, easily quantitated sequence target; thus, primer sets can be initially validated by using dilutions of linearized plasmid DNA. Plasmids are selected to carry the viral insert in mRNA sense orientation with respect to the T7 promoter sequence. Plasmids will be linearized by restriction digestion using an appropriate enzyme that cleaves in the polylinker region downstream of the insert. Where the cloned target sequence is predicted to contain the available restriction sites, a suitable unique restriction site is introduced via the PCR primer used during cloning of the respective target. Purified linearized plasmid DNA is serially diluted in background DNA (human placenta DNA, Sigma) to result in 5×10^5 , $5 \times$

10⁴, 5 x 10³, 5 x 10², 5 x 10¹, and 5 x 10⁰ copies per assay.

Once optimal primer sets for detection of all relevant
5 enteroviruses are identified, the sensitivity of the
entire procedure including RNA extraction and reverse
transcription is assessed. Synthetic RNA transcripts of
each target sequence are generated from the linearized
10 plasmid DNA using T7 RNA polymerase. Transcripts are
serially diluted in background RNA relevant to the
primary hypothesis (e.g., ALS, normal spinal cord RNA).
Individual dilutions representing 5 x 10⁵, 5 x 10⁴, 5 x
10³, 5 x 10², 5 x 10¹, and 5 x 10⁰ copies per assay in a
background of 25 ng/ul total RNA are extracted with Tri-
15 Reagent, reverse transcribed, and then subjected to Mass-
Tag PCR.

Specificity of the identified primer sets relevant to
multiplexing can be assessed by using one desired primer
20 set in conjunction with its respective target sequence at
5 times threshold concentration in the presence of all
other, potentially cross-reacting, target sequences at a
10²-, 10⁴- and 10⁶-fold excess.

25 PCR amplification is performed using photocleavable mass
tagged primers in the presence of a biotinylated
nucleotide (e.g. Biotin-16-dUTP, Roche) to allow removal
of excess primer after PCR. Amplification products will
be purified from excess primer by binding to a
30 streptavidin-coated solid phase such as streptavidin-
Sephacrose (Pharmacia) or streptavidin coated magnetic
beads (Dynal) via biotin-streptavidin interaction.

Molecular mass tags can be made cleavable by irradiation with near UV light (~350 nm), and the released tags introduced by either chromatography or flow injection into a pneumatic nebulizer for detection in an atmospheric pressure chemical ionization mass spectrometer. Alternatively, to increase the specificity of detection by analyzing only PCR products of the expected size range, the mass tagged amplicons, can be size-selected (without the requirement for biotinylated nucleotides) using HPLC.

Multiplex Detection and Identification of Enteroviral Transcripts

A method that allows simultaneous detection of a broad range of enteroviruses with similar sensitivity was developed. A series of 4 primer sets were identified in the 5'-UTR predicted to detect all enteroviruses. These can be combined into two or perhaps even one mixed set for multiplex PCR. Two different genomic regions, 5'-UTR and polymerase, are targeted with independent primer panels, in order to confirm presence of enterovirus infection.

Once the presence of enteroviral sequences are confirmed using broad range primer sets, a different primer set is used to discriminate amongst the various enteroviral species. Whereas broad range primers are selected from the highly conserved 5'-UTR and polymerase 3D gene regions, the primer sets used to identify the enterovirus species target the most divergent genomic regions in VP3 and VP1.

Limitations must be considered in that although cerebral spinal fluid is unlikely to contain more than a single enterovirus (the virus responsible for clinical disease in an individual patient), individual stool samples may contain several enteroviruses. It is important, therefore, that assays not favor amplification or detection of one viral species over another. Second, multiplexing can result in loss of sensitivity. Thus, panels should be assessed for sensitivity (and specificity) with addition of new primer sets.

Direct MS-sequencing of PCR Amplified Enteroviral Transcripts for virus species identification

MALDI MS has been explored widely for DNA sequencing; however, this approach requires that the DNA sequencing fragments be free from alkaline and alkaline earth salts, as well as other contaminants, to ensure accurate measurements of the masses of the DNA fragments. We explored a novel MS DNA sequencing method that generates Sanger-sequencing fragments using biotinylated dideoxynucleotides labeled with mass tags.

The ability to distinguish various nucleotide bases in DNA using mass spectrometry is dependent on the mass differences of the DNA ladders in the mass spectra. Smith et al. have shown that using dye labeled ddNTP paired with a regular dNTP to space out the mass difference can increase the detection resolution in a single nucleotide extension assay (10). Preliminary studies using biotin-11-dd(A, C, G)TPs and biotin-16-

ddTTP, indicated that the smallest mass difference between any two nucleotides is 16 daltons. To enhance the ability to distinguish peaks in the sequencing spectra, the mass separation of the individual ddNTPs can be increased by systematically modifying the biotinylated dideoxynucleotides by incorporating mass linkers assembled using 4-aminomethyl benzoic acid derivatives. The mass linkers can be modified by incorporating one or two fluorine atoms to further space out the mass differences between the nucleotides. The structures of the newly designed biotinylated ddNTPs are shown in Fig. 7. Linkers are attached to the 5 position on the pyrimidine bases (C and T), and to the 7 position on the purines (A and G) to facilitate conjugation with biotin. It has been established that modification of these positions on the bases in the nucleotides, even with bulky energy transfer (ET) fluorescent dyes, still allows efficient incorporation of the modified nucleotides into the DNA strand by DNA polymerase (24, 31). Biotin and the mass linkers are considerably smaller than the ET dyes, ameliorating difficulties in incorporation of ddNTP-linker-biotin molecules into DNA strands in sequencing reactions.

The DNA sequencing fragments that carry a biotin at the 3'-end are made free from salts and other components in the sequencing reaction by capture with streptavidin-coated magnetic beads. Thereafter, the correctly terminated biotinylated DNA fragments are released and loaded onto the mass spectrometer. Results indicate that MS can produce high resolution of DNA-sequencing fragments, fast separation on microsecond time scales,

and eliminate the compressions associated with gel electrophoresis.

Amplification products obtained by PCR with broad range
5 5'-UTR or polymerase 3D primer sets can be used as
template. Sequencing permits discrimination between bona
fide enteroviral amplification products and artifacts.
Where analysis of the semi-divergent sequence region
located toward the 3'-end of the 5'-UTR region is
10 inadequate for speciation, targeting the more divergent
VP3 and/or VP1 regions is preferred.

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Example 2Multiplex Mass Tag PCR Detection of Respiratory Pathogens*Background and Significance*

5

The advent of SARS in 2003 poignantly demonstrated the urgency of establishing rapid, sensitive, specific, inexpensive tools for differential laboratory diagnosis of infectious diseases. Through unprecedented global collaborative efforts, the causative agent was rapidly implicated and characterized, facilitating development of serologic and molecular assays for infection, and containment of the outbreak. Nonetheless, as the northern hemisphere entered the winter season of 2004, the diagnosis of SARS still rested on clinical and epidemiological as well as laboratory criteria.

Methods for cloning nucleic acids of microbial pathogens directly from clinical specimens offer new opportunities to investigate microbial associations in diseases. The power of these methods is not only sensitivity and speed but also the potential to succeed where methods for pathogen identification through serology or cultivation may fail due to absence of specific reagents or fastidious requirements for agent replication.

Various methods are employed or proposed for cultivation-independent characterization of infectious agents. These can be broadly segregated into methods based on direct analysis of microbial nucleic acid sequences, direct analysis of microbial protein sequences, immunological

systems for microbe detection, and host response profiling. Any comprehensive armamentarium should include most, if not all, of these tools. Nonetheless, classical methods for microbiology remain important. Indeed, the
5 critical breakthrough during the SARS outbreak was the cultivation of the agent in tissue culture.

Real-time PCR methods have significantly changed diagnostic molecular microbiology by providing rapid,
10 sensitive, specific tools for detecting and quantitating genetic targets. Because closed systems are employed, real-time PCR is less likely than nested PCR to be confounded by assay contamination due to inadvertent aerosol introduction of amplicon/positive control/cDNA
15 templates that can accumulate in diagnostic laboratories. The specificity of real time PCR is both a strength and a limitation. Although the potential for false positive signal is low so is the utility of the method for screening to detect related but not identical genetic
20 targets. Specificity in real-time PCR is provided by two primers (each approximately 20 matching nucleotides (nt) in length) combined with a specific reporter probe of about 27 nt. The constraints of achieving hybridization at all three sites may confound detection of diverse,
25 rapidly evolving microbial genomes such as those of single-stranded RNA viruses. These constraints can be compensated in part by increasing numbers of primer sets accommodating various templates. However, because real-time PCR relies on fluorescent reporter dyes, the
30 capacity for multiplexing is limited to the number of emission peaks that can be unequivocally separated. At present up to four dyes can be identified simultaneously.

Although the repertoire may increase, it will unlikely to change dramatically.

To address the need for enhanced multiplex capacity in diagnostic molecular microbiology we have established a PCR platform based on mass tag reporters that are easily distinguished in MS as discrete signal peaks. Major advantages of the PCR/MS system include: (1) hybridization to only two sites is required (forward and reverse primer binding sites) vs real time PCR where an intermediate third oligonucleotide is used (probe binding site); this enhances flexibility in primer design; (2) tried and proven consensus PCR primers can be adapted to PCR/MS; this reduces the time and resources that must be invested to create new reagents and assay controls; (3) the large repertoire of tags allows highly multiplexed assays; additional tags can be easily synthesized to allow further complexity; and (4) sensitivity of real time PCR is maintained. We view PCR/MS as a tool with which to rapidly screen clinical materials for the presence of candidate pathogens. Thereafter, targeted secondary tests, including real time PCR, can be used to quantitate microbe burden and pursue epidemiologic studies.

25

Preliminary Data

We have developed bioinformatic tools to facilitate sequence alignments, motif identification, and primer design; established banks of viral strains, cDNA templates, and primers; and built relationships with collaborators in national and global public health

laboratory networks that provide access to data, organisms, sera, and cDNAs that facilitate assay development and validation. Over the past two years we have integrated PCR and MS into a stable and sensitive digital assay platform similar in sensitivity and efficiency to real time PCR but with the advantages of simultaneous detection and discrimination of multiple targets. Using the 4 tags created for DNA sequencing we initially tested the method with flavivirus and bunyavirus targets as a proof of principle for an encephalitis project. The collaboration was later expanded to include two industrial partners: QIAGEN GmbH, a partner with a large validated library of proprietary photocleavable mass tags (Masscode™) and expertise in manufacture and commercial distribution, and Griffin Analytical Technologies, a partner actively engaged in design and fabrication of low cost portable MS instruments for field applications.

20 *Selection of APCI LCMS Platform*

Mass spectrometry is a rapid, sensitive method for detection of small molecules. With the development of Ionization techniques such as matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), MS has become a indispensable tool in many areas of biomedical research. Although these ionization methods are suitable for the analysis of bioorganic molecules, such as peptides and proteins, improvements in both detection and sample preparation will be required before mass spectrometry can be used to directly detect long DNA fragments. A major confound in exploiting MS for genetic

investigation has been that long DNA molecules are fragmented during the analytic process. The mass tag approach we have developed overcomes this limitation by detecting small stable mass tags that serve as signatures
5 for specific DNA sequences rather than the DNA sequences themselves.

We have explored the kinetics of photocleavable primer conjugation. Ionization and detection of the photocleaved
10 mass tags have been extensively characterized using atmospheric pressure chemical ionization (APCI) as the ionization source while using a single quadrupole mass spectrometer as the detector (Jingyue et al. , Kim et al. 2003; Kokoris et al. 2000). Because buffer and inorganic
15 salts impact ionization efficiency, performance in ESI was determined to be critically dependent upon sample preparation conditions. In MALDI, matrix must be added prior to sample introduction into the mass spectrometer, which is a time consuming step that requires costly
20 sample spotting instrumentation. Similary, speed is often limited by the need to search for an ideal irradiation spot to obtain interpretable mass spectra.

In contrast, APCI is much more tolerant of residual
25 inorganic salts (than ESI) and does not require mixing with matrix to prepare crystals on a target plate. Thus, mass tag solutions can be injected directly into the MS via a Liquid Chromatography (LC) delivery system. Since mass tags ionize well under APCI conditions and have
30 small mass values (less than 800 amu), they are detected with high sensitivity (< 5 femtomolar limit of detection) with the APCI-Quadrupole LCMS platform.

Methods for synthesis and APCI-MS analysis of mass tags coupled to DNA fragments are illustrated in Fig. 8 where precursors are (a) acetophenone; (b) 4-fluoroacetophenone; (c) 3-methoxyacetophenone; and (d) 3,4-dimethoxyacetophenone.

Upon nitration and reduction, the photoactive tags are produced and used to code for the identity of different primer pairs. An example for photocleavage and detection of four tags is shown in Figure 9 which shows APCI mass spectra for four mass tags after from the corresponding primers (mass tag # 1, 2-nitrosoacetophenone, m/z 150; mass tag # 2, 4-fluoro-2-nitrosoacetophenone, m/z 168; mass tag # 3, 5-methoxy-2-nitrosoacetophenone, m/z 180; mass tag # 4, 4,5-dimethoxy-2-nitrosoacetophenone, m/z 210). The four mass tag-labeled primers were mixed together and the mixture was irradiated under UV light (λ -340 nm) for 5 seconds, introduced into an APCI mass spectrometer and analyzed for the four masses to produce the above spectrum. The peak with m/z of 150 is mass-tag 1, 168 is mass-tag 2, 180 is mass-tag 3 and 210 is mass-tag 4. The mechanism for release of these tags from DNA is shown in Fig. 10 - Four mass tag-labeled DNA molecules (Bottom) Chemical structures of the corresponding photocleaved mass tags (2-nitrosoacetophenone, 4-fluoro-2-nitrosoacetophenone, 5-methoxy-2-nitrosoacetophenone and 4,5-dimethoxy-2-nitrosoacetophenone) after UV irradiation at 340 nm. This result indicates that the 4 compounds designed as mass tags are stable and produce discrete high-resolution digital data in an APCI mass spectrometer. The unique m/z from each mass tag

translates to the identity of a viral sequence. In a recent collaboration with Qiagen, which has used a library of mass tags to discriminate up to 25 SNPs (Kokoris et al. 2000), we have significantly expanded the
5 number of the mass tags.

Establishment of a PCR/MS Assay for Respiratory Pathogens

During the SARS 2003 Beijing outbreak we established a
10 specific and sensitive real time PCR assay for SARS-CoV (Zhai et al, 2004). The assay was extended to allow simultaneous detection of SARS-CoV as well as human coronaviruses OC43 and 229E in light of recent data from China suggesting the potential for coinfection and
15 increased morbidity (Fig. 11). This human coronavirus assay (3 viral genes and 1 housekeeping gene) exhausted the repertoire of fluorescent tags with which to pursue multiplex real time PCR analysis of clinical materials. The importance of extending rapid molecular assays to
20 include other respiratory pathogens is reinforced by the reappearance of SARS in China and reports of a new highly virulent influenza virus strain in Vietnam.

To build a more comprehensive respiratory pathogen
25 surveillance assay we adapted the human coronavirus primers to the PCR/MS platform, and added reagents required to detect other relevant microbes. Influenza A virus was included through a set of established primer sequences obtained through Georg Pauli (Robert Koch
30 Institute, Germany; Schwaiger et al 2000). For the bacterial pathogen *M. pneumoniae* we also used unmodified primer sequences published for real time PCR (Welte et al

2003) to evaluate their use on the PCR/MS platform. Using a panel of mass tags developed by QIAGEN, experiments were performed demonstrating the feasibility of detecting several respiratory pathogens in a single multiplexed
5 assay on the PCR/MS platform.

The current Masscode™ photocleavable mass tag repertoire comprises over 80 tags. Fig. 12 demonstrates the specificity of the mass tag detection approach in an
10 example where 58 different mass tags conjugated to oligonucleotides via a photocleavable linkage were identified after UV cleavage and MS. Each of the 10 primers for the 5-plex assay (SARS-CoV, CoV-229E, CoV-OC43, Influenza A virus, and *M. pneumoniae*) was
15 conjugated to a different mass tag such that the identity of a given pathogen was encoded by a specific binary signal (e.g. SARS-CoV, forward primer, 527 amu; reverse primer 666 amu; see Fig. 13B).

20 The presence of mass tags did not impair performance of primers in PCR and yielded clear signals for all 5 agents (Fig. 13A, 13B - Singleplex mass tag PCR for (1) Influenza A virus matrix protein (618 amu fwd-primer, 690 amu rev-primer), human coronaviruses (2) SARS (527/666),
25 (3) 229E (670/558), (4) OC43 (686/548), and the bacterial agent (5) *M. pneumoniae* (602/614). (6) 100 bp ladder). No noise was observed using unmodified or mass tag-modified primer sets in a background of 125 ng of normal total human DNA per assay (Fig. 13C). In subsequent experiments
30 we extended the respiratory pathogen panel to include respiratory syncytial virus groups A and B. Non-optimized pilot studies in this 7-plex system indicated a detection

threshold of <500 molecules. As a test of feasibility for PCR/MS detection of coinfection, mixtures of DNA templates representing two different pathogens were analyzed successful detection of two targets confirmed
5 the suitability of this technology for clinical applications where coinfection may be critical to pathogenesis and epidemiology.

Establishment of a platform for portable MS

10

Griffin has developed a portable mass spectrometer that is roughly the size of a tower computer (including vacuum system), weighs less than 50 lbs, and consumes ~150 W depending on operating conditions. This system has a mass
15 range of 400 Da with unit mass resolution. It has been used to detect part-per-trillion level atmospheric constituents. Figure 14 shows a representative spectrum of methyl salicylate collected on a miniature cylindrical ion trap mass analyzer coupled to a corona discharge
20 ionization source (data collected in Prof. R. G. Cooks research laboratory at Purdue University). This data demonstrates the feasibility of using this type of instrumentation to detect the mass tags of interest as well as the specificity of the ionization source. Fig. 14
25 shows mass spectrum representative of data collected using a miniature cylindrical ion trap mass analyzer coupled with a corona discharge ionization source.

Figure 15 shows a mass spectrum of perfluoro-
30 dimethylcyclohexane collected on a prototype atmospheric sampling glow discharge ionization (ASGDI) source. ASGDI is an external ionization source related to the APCI

source discussed here.

Experimental Design

5 Labeled amplification products are generated during PCR
amplification with mass tagged primers. After isolation
from non-incorporated primers by binding to silica in
Qiagen 96-well or 384-well PCR purification modules,
products are eluted into the injection module of the
10 mass-spectrometer. The products traverse the path of a UV
light source prior to entering the nebulizer, releasing
photocleavable tags (one each from the forward and
reverse primer). Mass tags are then ionized. Analysis of
the mass code spectrum defines the pathogen composition
15 of the specimen.

A non-comprehensive list of target pathogens is listed in
Tables 2 and 3. Forward and reverse primer pairs for
pathogens listed in Table 2 are (reading from top to
20 bottom starting with RSV-A and ending with M.
Pneumoniae), SEQ ID NOS:1 and 2, 3 and 4, 9 and 10, 21
and 22, 23 and 24, 26 and 27, and 49 and 50.

Table 2: Respiratory Panel Mass-Tag Primers				
Pathogen	Forward primer	Sequence	Reverse primer	Sequence
RSV A	RSA-U1137	AgATCAACTTCTgTC ATCCAgCAA	RSV-L1192	gCACATCATAATTAggAg TATCAAT
RSV B	RSB-U1248	AAGATgCAAATCAT AAATTCACAggA	RSV-1318	TgATATCCAgCATCTTTA AgTATCTTTATAgTg
Influenza A (N1)				
Influenza A (N2)				
Influenza A (M)	AM-U151	CATggAATggCTAAA. gACAAGACC	AM-L397	AAGTgCACCAgCAGAATA ACTgAg
Influenza B				
SARS-CoV	CIID-28891F	AAG CCT CgC CAA AAA CgT AC	CIID-29100R	AAG TCA gCC ATg TTC CCg AA
229E-CoV	Taq-Co22-418F	ggC gCA AgA ATT CAg AAC CA	Taq-Co22-636R	TAA gAg CCg CAg CAA CTg C
OC43-CoV	Taq-Co43-270F	TgT gCC TAT TgC ACC Agg AgT	Taq-Co43-508R	CCC gAT CgA CAA TgT CAg C
Metapneumo virus				
Parainfluenza 1				
Parainfluenza 2				
Parainfluenza 3				
Parainfluenza 4				
<i>M. pneumoniae</i>	MTPM1	CCAACCAAACAACA ACgTTCA	MTPM2	ACCTTgACTggAggCCgTT A
<i>L. pneumophi</i>				

lae				
C. pneumonia e				

Design and Synthesis of Primers

5 Primers are designed using the same approach as employed for the 7-plex assay. Available sequences are be extracted from GenBank. Conserved regions suitable for primer design are identified using standard software programs as well as custom software (patent application XYZ). Primer
10 properties can be assessed by commercial primer selection software including OLIGO (Molecular Biology Insights), Primer Express (PE Applied Biosystems), and Primer Premiere (Premiere Biosoft International). Primers are evaluated for signal strength and specificity against a
15 background of total human DNA.

Isolation and Cloning of Template Standards

Targeted genes can be cloned into the transcription
20 vector pGEM-Teasy (Invitrogen) by conventional RT-PCR cloning methods. Quantitated plasmid standards are used in initial assay establishment. Thereafter, RNA transcripts generated by in vitro transcription, quantitated and diluted in a background of random human
25 RNA (representing brain, liver, spleen, lung and placenta in equal proportions) are employed to establish sensitivity and specificity parameters of RT-PCR/MS assays. One representative isolate for each targeted

pathogen/gene is used during initial establishment of the assay.

Inherent in the exquisite sensitivity of PCR is the risk of false positive results due to inadvertent introduction of synthetic templates such as those comprising positive control and calibration reagents, and so calibration reagents are preferred components of kits. Thus, to allow recognition of control vs authentic, natural amplification products, calibration reagents are modified by introducing a restriction enzyme cleavage site in between the primer binding sites through site directed mutagenesis. This approach has been employed in projects concerned with epidemiology of viral infection in various chronic diseases including Bornaviruses in neuropsychiatric disease (NIH/MH57467), measles virus in autism (CDC/American Academy of Pediatrics), and enteroviruses in type I diabetes mellitus (NIH/AI55466).

Multiplex Assay Using Cloned Template Standards

Initially, the performance of individual primer sets with unmodified primers is tested. Amplification products in these single assays can be detected by gel electrophoresis. This strategy will not serve for multiplex assays because products of individual primer sets will be similar in size i.e. <300 bp. Thus, after confirmation of performance in single assays, mass tagged primers are generated for multiplex analyses. All assays are first optimized for PCR using serial dilutions of plasmid DNA, and then for RT-PCR using serial dilutions of synthetic transcripts. A multiplex assay is considered

successful if it detects all target sequences at a sensitivity of 50 copies plasmid DNA per assay and 100 copies RNA per assay. Successful multiplex assay performance includes detection of all permutative combinations of two agents to ensure the feasibility of diagnosing simultaneous infection.

Optimizing Multiplex Assay Using Cell Culture Extracts

After establishing performance parameters with calibrated synthetic reagents, cell culture extracts of authentic pathogens are used. Performance of assays with RNA extracted using readily available commercial systems that do or do not include organic solvents (e.g, Tri-Reagent vs RNeasy) is assessed. A protocol disclosed here employs Tri-Reagent. Similarly, although Superscript reverse transcriptase (Invitrogen) and HotStart polymerase (QIAGEN) can be used, performance of ThermoScript RT (Invitrogen) at elevated temperature can be assessed, as are single-step RT-PCR systems like the Access Kit (Promega). To optimize efficiency where clinical material mass is limited and to reduce the complexity of sample preparation, both viral and bacterial agents can be identified using RT-PCR. Where an agent is characterized by substantive phylogenetic diversity, cell culture systems should include at least three divergent isolates of each pathogen

Sample Processing

Samples may be obtained by nasal swabs, sputum and lavage specimens will be spiked with culture material to optimize recovery methods for viral as well as bacterial

agents.

Portable APCI MS instruments to support multiplex PCR/MS platform

5 The multiplex mass tag approach is well-suited to
implementation on a miniaturized MS system, as the
photocleavable mass tags are all relatively low in
molecular weight (<500 Da.), and hence the constraints on
10 the mass spectrometer in terms of mass range and mass
resolution are not high. The technical challenge
associated with this approach is the development of an
atmospheric-pressure chemical ionization (APCI) source
for use on a miniaturized MS to generate the mass tag
15 ions. Such a source has been coupled with a miniaturized
MS in an academic setting.

Detection of NIAD Category A, B, and C Priority Agents

20 Using the same approach as outlined for respiratory
pathogen detection, a multiplex assay for detection of
selected NIAD Category A, B, and C priority agents can be
created (Table 3). Primers and PCR conditions for several
agents are already established and can be adapted to the
25 PCR/MS platform.

5

Table 3: NIAD Priority Agents	
B. anthracis	
Dengue viruses	
West Nile virus	
Japanese encephalitis virus	
St. Louis encephalitis virus	
Yellow Fever virus	
La Crosse virus	
California encephalitis virus	
Rift Valley Fever virus	
CCHF virus	
VEE virus	
EEE virus	
WEE virus	
Ebola virus	
Marburg virus	
LCMV	
Junin virus	
Machupo virus	
Variola virus	

Example 3*Background*

5 Efficient laboratory diagnosis of infectious diseases is increasingly important to clinical management and public health. Methods for direct detection of nucleic acids of microbial pathogens in clinical specimens are rapid, sensitive and may succeed where fastidious requirements
10 for agent replication confound cultivation. Nucleic acid amplification systems are indispensable tools in HIV and HCV diagnosis, and are increasingly applied to pathogen typing, surveillance, and diagnosis of acute infectious disease. Clinical syndromes are only infrequently
15 specific for single pathogens; thus, assays for simultaneous consideration of multiple agents are needed. Current multiplex assays employ gel-based formats where products are distinguished by size, fluorescent reporter dyes that vary in color, or secondary enzyme
20 hybridization assays. Gel-based assays are reported that detect 2-8 different targets with sensitivities of 2-100 pfu or <1-5 pfu, depending on whether amplification is carried out in a single or nested format, respectively (Ellis and Zambon 2002, Coiras et al. 2004).
25 Fluorescence reporter systems achieve quantitative detection with sensitivity similar to nested amplification; however, their capacity to simultaneously query multiple targets is limited to the number of fluorescent emission peaks that can be unequivocally
30 separated. At present up to four fluorescent reporter dyes are detected simultaneously (Vet et al. 1999, Verweij et al. 2004). Multiplex detection of up to 9

pathogens was achieved in hybridization enzyme systems; however, the method requires cumbersome post-amplification processing (Gröndahl et al. 1999).

5 To address the need for sensitive multiplex assays in diagnostic molecular microbiology we created a polymerase chain reaction (PCR) platform wherein microbial gene targets are coded by 64 distinct mass tags. Here we describe this system, mass tag PCR, and demonstrate its
10 utility in differential diagnosis of respiratory tract infections.

Oligonucleotide primers for mass tag PCR were designed to detect the broadest number of members for a given
15 pathogen species through efficient amplification of a 50-300 basepair product. In some instances we selected established primer sets; in others we employed a software program designed to cull sequence information from GenBank, perform multiple alignments, and maximize
20 multiplex performance by selecting primers with uniform melting temperatures and minimal cross-hybridization potential. Primers, synthesized with a 5' C6-spacer and aminohexyl modification, were covalently conjugated via a photocleavable linkage to small molecular weight tags
25 (Kokoris et al. 2000) to encode their respective microbial gene targets. Forward and reverse primers were labeled with differently sized tags to produce a dual code for each target that facilitates assessment of signal specificity.

30

Microbial gene target standards for sensitivity and specificity assessment were cloned by PCR using cDNA

template obtained by reverse transcription of extracts from infected cultured cells or by assembly of overlapping synthetic polynucleotides. Cloned standards representing genetic sequence of the targeted microbial pathogens were diluted in 12.5 ug/ml human placenta DNA (Sigma, St. Louis, MO, USA) and subjected to multiplex PCR amplification using the following cycling protocol: 9x C for X sec., 55 C for X sec., 72 C for X sec.; 50 cycles, MJ PTC200 (MJ Research, Waltham, MA, USA). Amplification products were purified using QIAquick 96 PCR purification cartridges (Qiagen, Hilden, Germany) with modified binding and wash buffers (RECIPES). Mass tags of the amplified products were analyzed after ultraviolet photolysis and positive-mode atmospheric pressure chemical ionization (APCI) by single quadrupole mass spectrometry. Figure 1 indicates discrimination of individual microbial targets in a 21-plex assay comprising sequences of 16 human pathogens. The threshold of detection met or exceeded 500 molecules corresponding in sensitivity to less than 0.1 TCID₅₀/ml (0.001 TCID₅₀/assay), in titered cell culture virus of coronaviruses as well as parainfluenza viruses (data not shown). For 19 of 21 microbial targets the detection threshold was less than 100 molecules (Table 4).

We next analyzed samples from individuals with respiratory infection using a larger panel comprising 30 gene targets (26 pathogens). Mass Tag PCR correctly identified infection with respiratory syncytial, human parainfluenza, SARS corona, adeno, entero, metapneumo and influenza viruses (Table 4 and Figure 16). A smaller panel comprising 18 gene targets (18 central nervous

system pathogens) was used to analyze cerebrospinal fluid from individuals with meningitis or encephalitis. Two of four cases of West Nile virus encephalitis were identified. Fifteen of seventeen cases of enteroviral
5 meningitis were detected representing serotypes CV-B2, CV-B3, CV-B5, E-6, E-11, E-13, E-18, and E-30.

Our results indicate that mass tag PCR is a useful method for molecular characterization of microflora. Sensitivity
10 is similar to real time PCR assays but with the advantage of allowing simultaneous screening for several candidate pathogens. Potential applications include differential diagnosis of infectious diseases, blood product surveillance, forensic microbiology, and biodefense.

15
Figure 16 shows the sensitivity of 21-plex mass tag PCR. Dilutions of cloned gene target standards (10 000, 1 000, 500, 100 molecules/assay) diluted in human placenta DNA were analyzed by mass tag PCR. Each reaction mix
20 contained 2x Multiplex PCR Master Mix (Qiagen), the indicated standard and 42 primers at 1X nM concentration labeled with different mass tags. Background in reactions without standard (no template control, 12.5 ng human DNA) was subtracted and the sum of Integrated Ion Current for
25 both tags was plotted.

Figure 17 shows analysis of clinical specimens. (A) Respiratory infection; (B) Encephalitis. RNA from clinical specimens was extracted by standard procedures
30 and reverse transcribed into cDNA (Superscript RT system, Invitrogen, Carlsbad, CA; 20 ul volume). Five microliter of reaction was then subjected to mass tag PCR. (A)

Detection of Influenza A (H1N1), RSV-B, SARS-CoV, HPIV-3, HPIV-4, and ENTERO using a 31-plex assay including 64 primers targeting Influenza A virus (FLUAV) matrix gene, and for typing H1, H2, H3, H5, N1, and N2 sequence, as
5 well as influenza B virus (FLUBV), respiratory syncytial virus (RSV) groups A and B, human coronaviruses 229E, OC43, and SARS (HCoV-229E, -OC43, and -SARS), human parainfluenza virus (HPIV) types 1, 2, 3, and 4 (groups A and B combined), metapneumovirus, enteroviruses (EV,
10 targeting all serogroups), adenoviruses (HAdV, targeting all serogroups), Mycoplasma pneumoniae, Chlamydia pneumoniae, Legionella pneumophila, Streptococcus pneumoniae, Haemophilus influenzae, Human herpesvirus 1 (HHV-1, Herpes simplex virus), Human herpesvirus 3 (HHV-
15 3; Varicella-zoster virus), Human herpesvirus 5 (HHV-5, Human cytomegalovirus), Human immunodeficiency virus 1 (HIV-1) and Human immunodeficiency virus 1HIV-2. (B) Detection of ENTERO XX, YY, and ZZ using an 18-plex assay including 36 primers targeting FLUAV matrix gene, H1, H2,
20 H3, H5, N1, and N2 sequence, FLUBV, HCoV 229E, OC43, and SARS, EV, HAdV, HHV-1, -3, and -5, HIV-1, and -2, measles virus (MEV), West Nile virus (WNV), St. Louis virus (SLEV), S. pneumoniae, H. influenzae, and Neisseria meningitides.

25

Influenza A Matrix	Influenza A N1	Influenza A N2	Influenza A HA1	Influenza A HA2	Influenza A HA3	Influenza A HA5	Influenza B HA	RSV Group A	RSV group B	Metapneumo virus
100	100	100	100	100	100	100	500	100	100	100
CoV-SARS	CoV-OC43	CoV-229E	HPiV-1	HPiV-2	HPiV-3	C. pneumoniae	M. pneumoniae	L. pneumophila	Enterovirus (genus)	Adenovirus (genus)
100	100	100	100	100	100	100	100	100	5 000	5 000

Table 4. Sensitivity of 22-plex mass tag PCR. Numbers in cells indicate target copy threshold.

Example 4*Multiplex PCR*

5

Conventional multiplex PCR assays are established, however, none allow sensitive detection of more than 10 genetic targets. The most sensitive of these assays, real time PCR, is limited to four fluorescent reporter dyes. 10 Gel based systems are cumbersome and limited to visual distinction of products that differ by 20 bp; multiplexing is restricted to the number of products that can be distinguished at 20 bp intervals within the range of 100 to 250 bp (amplification efficiency decreases with 15 larger products); nesting or Southern hybridization is required for high sensitivity. A 9-plex assay has been achieved using hybridization capture enzyme assay.

Disclosed here are panels of nucleic acid sequences to be 20 used in assays for the detection of infectious agents. The sequences include primers for polymerase chain reaction, enzyme sites for initiating isothermal amplification, hybridization selection of nucleic acid targets, as well as templates to serve as controls for 25 validation of these assays. This example focuses on the use of these panels for multiplex mass tag PCR applications. Nucleic acid databases were queried to identify regions of sequence conservation within viral and bacterial taxa wherein primers could be designed that 30 met the following criteria: (i) the presence of motifs required to create specific or low degeneracy PCR primers that targeted all members of a microbial group (or

subgroup); (ii) T_m of 59-61 C; (iii) GC content of 48-60%; (iv) length of 18-24 bp; (v) no more than three consecutive identical bases; (vi) 3 or more G and/or C residues in the 5'-hexamer; (vii) less than 3 G and/or C residues in the 3'-pentamer; (viii) no propensity for secondary structure (stem-loop) formation; (ix) no inter-primer complementarity that could predispose to primer-dimer formation; (x) amplification of an 80-250 bp region with no or little secondary structure at 59-61 C. Primers meeting these criteria were then evaluated empirically for equal performance in context of the respective multiplex panel. In the event that no ideal primer candidates could be identified, primers that did not meet one or more of these criteria were synthesized and evaluated for appropriate performance. Those that yielded 80-250 bp amplification products, had T_m of 59-61 C, and showed no primer-dimer artifacts were selected for inclusion into panels.

As a proof-of-principle we designed a panel of primers for detection of 31 target sequences of respiratory pathogens (25-plex respiratory panel) and demonstrated successful detection of all potential targets in a 25-plex PCR reaction. Detection of amplification products was achieved through use of the MASSCODE[®] technology. Individual primers were conjugated with a unique masscode tag through a photocleavable linkage. Photocleavage of the masscode tag from the purified PCR product and mass spectrometric analysis identifies the amplified target through the two molecular weights assigned to the forward and reverse primer. Primer panels focus on groups of

infectious pathogens that are related to differential
diagnosis of respiratory disease, encephalitis, or
hemorrhagic fevers; screening of blood products;
biodefense; food safety; environmental contamination; or
5 forensics.

Example 5*Background and Significance*

5

The advent of SARS in 2003 poignantly demonstrated the urgency of establishing rapid, sensitive, specific, inexpensive tools for differential laboratory diagnosis of infectious diseases. Through unprecedented global
10 collaborative efforts, the causative agent was rapidly implicated and characterized, facilitating development of serologic and molecular assays for infection, and containment of the outbreak. Nonetheless, as the northern hemisphere entered the winter season of 2004, the
15 diagnosis of SARS still rests on clinical and epidemiological as well as laboratory criteria. The WHO SARS International Reference and Verification Laboratory Network met on October 22, 2003 to review the status of laboratory diagnostics in acute severe pulmonary disease.
20 Quality assurance testing indicated that false positive SARS CoV PCR results were infrequent in network labs. However, participants registered concern that current assays did not allow simultaneous detection of a wide range of pathogens that could aggravate disease or
25 themselves result in clinical presentations similar to SARS.

Methods for cloning nucleic acids of microbial pathogens directly from clinical specimens offer new opportunities
30 to investigate microbial associations in diseases. The power of these methods is not only sensitivity and speed

but also the potential to succeed where methods for pathogen identification through serology or cultivation may fail due to absence of specific reagents or fastidious requirements for agent replication.

5

Various methods are employed or proposed for cultivation-independent characterization of infectious agents. These can be broadly segregated into methods based on direct analysis of microbial nucleic acid sequences, direct
10 analysis of microbial protein sequences, immunological systems for microbe detection, and host response profiling. Any comprehensive armamentarium should include most, if not all, of these tools. Nonetheless, classical methods for microbiology remain important. Indeed, the
15 critical breakthrough during the SARS outbreak was the cultivation of the agent in tissue culture.

Real-time PCR methods have significantly changed diagnostic molecular microbiology by providing rapid,
20 sensitive, specific tools for detecting and quantitating genetic targets. Because closed systems are employed, real-time PCR is less likely than nested PCR to be confounded by assay contamination due to inadvertent aerosol introduction of amplicon/positive control/cDNA
25 templates that can accumulate in diagnostic laboratories. The specificity of real time PCR is both, a strength and a limitation. Although the potential for false positive signal is low so is the utility of the method for screening to detect related but not identical genetic
30 targets. Specificity in real-time PCR is provided by two primers (each approximately 20 matching nucleotides (nt)

in length) combined with a specific reporter probe of about 27 nt. The constraints of achieving hybridization at all three sites may confound detection of diverse, rapidly evolving microbial genomes such as those of single-stranded RNA viruses. These constraints can be compensated in part by increasing numbers of primer sets accommodating various templates. However, because real-time PCR relies on fluorescent reporter dyes, the capacity for multiplexing is limited to the number of emission peaks that can be unequivocally separated. At present up to four dyes can be identified simultaneously. Although the repertoire may increase, it will unlikely to change dramatically.

To address the need for enhanced multiplex capacity in diagnostic molecular microbiology we have established a PCR platform based on mass tag reporters that are easily distinguished in MS as discrete signal peaks. Major advantages of the PCR/MS system include: (1) hybridization to only two sites is required (forward and reverse primer binding sites) vs real time PCR where an intermediate third oligonucleotide is used (probe binding site); this enhances flexibility in primer design; (2) tried and proven consensus PCR primers can be adapted to PCR/MS; this reduces the time and resources that must be invested to create new reagents and assay controls; (3) the current repertoire of 60 tags allows highly multiplexed assays; additional tags can be easily synthesized to allow further complexity; and (4) sensitivity of real time PCR is maintained. A limitation of PCR/MS is that it is unlikely to provide more than a

semi-quantitative index of microbe burden. Thus, we view PCR/MS as a tool with which to rapidly screen clinical materials for the presence of candidate pathogens. Thereafter, targeted secondary tests, including real time
5 PCR, should be used to quantitate microbe burden and pursue epidemiologic studies.

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20 fragments. A major confound in exploiting MS for genetic investigation has been that long DNA molecules are fragmented during the analytic process. The mass tag approach we have developed overcomes this limitation by detecting small stable mass tags that serve as signatures
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Ionization and detection of the photocleaved mass tags have been extensively characterized using atmospheric
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10 contrast, APCI is much more tolerant of residual inorganic salts (than ESI) and does not require mixing with matrix to prepare crystals on a target plate. Thus, mass tag solutions can be injected directly into the MS via a Liquid Chromatography (LC) delivery system. Since
15 mass tags ionize well under APCI conditions and have small mass values (less than 800 amu), they are detected with high sensitivity (< 5 femtomolar limit of detection) with the APCI-Quadrupole LCMS platform.

20 Methods for synthesis and APCI-MS analysis of mass tags coupled to DNA fragments are illustrated in Figure 1 where precursors are (a) acetophenone; (b) 4-fluoroacetophenone; (c) 3-methoxyacetophenone; and (d) 3,4-dimethoxyacetophenone.

25

Upon nitration and reduction, the photoactive tags are produced and used to code for the identity of different primer pairs. An example for photocleavage and detection of four tags is shown in Figure 9. APCI mass spectra for
30 four mass tags after from the corresponding primers (mass tag # 1, 2-nitrosoacetophenone, m/z 150; mass tag # 2, 4-

fluoro-2-nitrosoacetophenone, m/z 168; mass tag # 3, 5-methoxy-2-nitrosoacetophenone, m/z 180; mass tag # 4, 4,5-dimethoxy-2-nitrosoacetophenone, m/z 210). The four mass tag-labeled primers were mixed together and the
5 mixture was irradiated under UV light ($\lambda \sim 340$ nm) for 5 seconds, introduced into an APCI mass spectrometer and analyzed for the four masses to produce the spectrum. The peak with m/z of 150 is mass-tag 1, 168 is mass-tag 2, 180 is mass-tag 3 and 210 is mass-tag 4.

10

The mechanism for release of these tags from DNA is shown in Fig. 10. Four mass tag-labeled DNA molecules (Bottom) Chemical structures of the corresponding photocleaved mass tags (2-nitrosoacetophenone, 4-fluoro-2-nitrosoacetophenone, 5-methoxy-2-nitrosoacetophenone and 4,5-dimethoxy-2-nitrosoacetophenone) after UV irradiation
15 at 340 nm.

This result indicates that the 4 compounds designed as
20 mass tags are stable and produce discrete high-resolution digital data in an APCI mass spectrometer. In the research plan described below, the unique m/z from each mass tag will translate to the identity of a viral sequence. Qiagen has developed a large library of more
25 than 80 proprietary masscode tags (Kokoris et al. 2000). Examples are shown in Figure 19.

Establishment of a PCR/MS assay for respiratory pathogens
During the SARS 2003 Beijing outbreak we established a
30 specific and sensitive real time PCR assay for SARS-CoV (Zhai et al, 2004). The assay was extended to allow

simultaneous detection of SARS-CoV as well as human coronaviruses OC43 and 229E in light of recent data from China suggesting the potential for coinfection and increased morbidity (Figure 11). This human coronavirus assay (3 viral genes and 1 housekeeping gene) exhausted the repertoire of fluorescent tags with which to pursue multiplex real time PCR analysis of clinical materials. The importance of extending rapid molecular assays to include other respiratory pathogens is reinforced by the reappearance of SARS in China and reports of a new highly virulent influenza virus strain in Vietnam.

To build a more comprehensive respiratory pathogen surveillance assay we adapted the human coronavirus primers to the PCR/MS platform, and added reagents required to detect other relevant microbes. Influenza A virus was included through a set of established primer sequences obtained through Georg Pauli (Robert Koch Institute, Germany; Schwaiger et al 2000). For the bacterial pathogen *M. pneumoniae* we also used unmodified primer sequences published for real time PCR (Welti et al 2003) to evaluate their use on the PCR/MS platform. Using a panel of mass tags developed by QIAGEN, pilot experiments were performed, demonstrating the feasibility of detecting several respiratory pathogens in a single multiplexed assay on the PCR/MS platform.

Subsequent to the 1999 West Nile Virus (WNV) outbreak in the U.S. we also built a real time PCR assay for differential diagnosis of flaviviruses WNV and St. Louis encephalitis virus - see Figure 20. Other validated tools

for broad range detection of NIAID priority agents include universal primer sets for detection of Dengue type 1, 2, 3, and 4; various primer sets detecting all members of the bunyamwera and California encephalitis serogroups of the bunyaviruses, see table 13, and not yet validated primer sets for detection of all six Venezuelan equine encephalitis virus serotypes developed for Molecular Epidemiology, AFEIRA/SDE. Brooks, TX.

10 The current Masscode photocleavable mass tag repertoire comprises over 80 tags. Figure 12 demonstrates the specificity of the mass tag detection approach in an example where 58 different mass tags conjugated to oligonucleotides via a photocleavable linkage were identified after UV cleavage and MS. Each of the 10 primers for the 5-plex assay (SARS-CoV, CoV-229E, CoV-OC43, Influenza A virus, and *M. pneumoniae*) was conjugated to a different mass tag such that the identity of a given pathogen was encoded by a specific binary signal (e.g. SARS-CoV, forward primer, 527 amu; reverse primer 666 amu; see Figure 13B). The presence of mass tags did not impair performance of primers in PCR and yielded clear signals for all 5 agents (Figures 13A, 13B). No noise was observed using unmodified or mass tag-modified primer sets in a background of 125 ng of normal total human DNA per assay (Figure 13C). In general, Figure 13 shows singleplex mass tag PCR for (1) Influenza A virus matrix protein (618 amu fwd-primer, 690 amu rev-primer), human coronaviruses (2) SARS (527/666), (3) 229E (670/558), (4) OC43 (686/548), and the bacterial agent (5) *M. pneumoniae* (602/614). (6) 100 bp ladder. In

subsequent experiments we extended the respiratory pathogen panel to include respiratory syncytial virus groups A and B. Non-optimized pilot studies in this 7-plex system indicated a detection threshold of <500 molecules (Figure 21). As a test of feasibility for PCR/MS detection of coinfection, mixtures of DNA templates representing two different pathogens were analyzed successful detection of two targets (Figure 21) confirmed the suitability of this technology for clinical applications where coinfection may be critical to pathogenesis and epidemiology.

Establishment of a platform for portable MS

Griffin has developed a portable mass spectrometer that is roughly the size of a tower computer (including vacuum system), weighs less than 50 lbs, and consumes ~150 W depending on operating conditions. This system has a mass range of 400 Da with unit mass resolution. It has been used to detect part-per-trillion level atmospheric constituents. Included below is a representative spectrum of methyl salicylate collected on a miniature cylindrical ion trap mass analyzer coupled to a corona discharge ionization source (data collected in Prof. R. G. Cooks research laboratory at Purdue University). This data demonstrates the feasibility of using this type of instrumentation to detect the mass tags of interest as well as the specificity of the ionization source. Figure 14 shows mass spectrum data representative of data collected using a miniature cylindrical ion trap mass analyzer coupled with a corona discharge ionization

source. Figure 15 shows a mass spectrum of perflouro-dimethclclcyclohexane collected on a prototype atmospheric sampling glow discharge ionization (ASGDI) source. ASGDI is an external ionization source related to the APCI
5 source proposed here.

Griffin has developed a mass spectrometer for field transportable use. Power consumption, weight, size, and
10 ease of use have been focus design points in the development of this instrument. It has not been designed specifically for interface to an atmospheric pressure ionization (API) source like the one proposed here for pathogen surveillance and discovery. Thus, our focus in
15 this proposal is directed toward the integration of an atmospheric pressure chemical ionization (APCI) source and the required vacuum, engineering, and software considerations associated with this integration.

20 *Experimental Design*

A cartoon of the assay procedure is shown in Figure 22. Labeled amplification products will be generated during PCR amplification with mass tagged primers. After
25 isolation from non-incorporated primers by binding to silica in Qiagen 96-well or 384-well PCR purification modules, products will be eluted into the injection module of the mass-spectrometer. The products traverse the path of a UV light source prior to entering the
30 nebulizer, releasing photocleavable tags (one each from the forward and reverse primer). Mass tags are then ionized. Analysis of the mass code spectrum defines the

pathogen composition of the specimen.

The repertoire of potential pathogens to be targeted during this project is listed in Table 13. Forward and reverse primer pairs for pathogens listed in Table 13 are (reading from top to bottom starting with RSV-A and ending with *M. Pneumoniae*), SEQ ID NOS:1 and 2, 3 and 4, 9 and 10, 21 and 22, 23 and 24, 26 and 27, and 49 and 50.

Table 13: Respiratory Panel Mass-Tag Primers				
Pathogen	Forward primer	Sequence	Reverse primer	Sequence
RSV A	RSA-U1137	AgATCAACTTCTgTCATCCA gCAA	RSV-L1192	gCACATCATAATTAggAgTATCAAT
RSV B	RSB-U1248	AAgATgCAAATCATAAAATTC ACAggA	RSV-1318	TgATATCCAgCATCTTTAAgTATCT TTATAgTg
Influenza A (N1)				
Influenza A (N2)				
Influenza A (M)	AM-U151	CATggAATggCTAAAgACAAG ACC	AM-L397	AAgTgCACCAgCAGAAATAACTgAg
Influenza B				
SARS-CoV	CIID-28891F	AAg CCT CgC CAA AAA CgT AC	CIID-29100R	AAg TCA gCC ATg TTC CCg AA
229E-CoV	Taq-Co22- 418F	ggC gCA AgA ATT CAg AAC CA	Taq-Co22- 636R	TAA gAg CCg CAg CAA CTg C
OC43-CoV	Taq-Co43- 270F	TgT gCC TAT TgC ACC Agg AgT	Taq-Co43- 508R	CCC gAT CgA CAA TgT CAg C
Metapneumovirus				
Parainfluenza 1				
Parainfluenza 2				
Parainfluenza 3				
Parainfluenza 4				
<i>M. pneumoniae</i>	MTPM1	CCAACCAAACAACgTTC A	MTPM2	ACCTTgACTggAggCCgTTA
<i>L. pneumophila</i>				
<i>C. pneumoniae</i>				

Design and synthesize primers

Missing primers will be designed using the same approach
5 as employed for the 7-plex assay. Available sequences
will be extracted from GenBank. Conserved regions
suitable for primer design will be identified using
standard software programs as well as custom software
(patent application XYZ). Primer properties will be
10 assessed by commercial primer selection software
including OLIGO (Molecular Biology Insights), Primer
Express (PE Applied Biosystems), and Primer Premiere
(Premiere Biosoft International). Non-tagged primers will
be synthesized, and performance assessed using cloned
15 target sequences as described in preliminary data.
Primers will be evaluated for signal strength and
specificity against a background of total human DNA.
Currently, 80% of primers perform as predicted by our
algorithms. Thus, to minimize delay we typically
20 synthesize multiple primer sets for similar genetic
targets and evaluate their performance in parallel.

Inherent in the exquisite sensitivity of PCR is the risk
of false positive results due to inadvertent introduction
25 of synthetic templates such as those comprising positive
control and calibration reagents. Calibration reagents
will be components of kits distributed to network
laboratories and customers. Thus, to allow recognition of
control vs authentic, natural amplification products, we
30 will modify calibration reagents by introducing a
restriction enzyme cleavage site in between the primer
binding sites through site directed mutagenesis. We have

used this approach in projects concerned with epidemiology of viral infection in various chronic diseases including Bornaviruses in neuropsychiatric disease (NIH/MH57467), measles virus in autism
5 (CDC/American Academy of Pediatrics), and enteroviruses in type I diabetes mellitus (NIH/AI55466).

Establish multiplex assay using cloned template standards

10 Before committing resources to generating mass tagged primers we will test the performance of individual primer sets with unmodified primers. Amplification products in these single assays will be detected by gel electrophoresis. This strategy will not serve for
15 multiplex assays because products of individual primer sets will be similar in size i.e., all will be <300 bp. Although individual products in multiplex assays could be resolved by sequence analysis our experience suggests it will be more cost effective to proceed directly to PCR/MS
20 analysis. Thus, after performance is confirmed in single assays we will generate mass tagged primers for multiplex analyses. All assays will be optimized first for PCR using serial dilutions of plasmid DNA, and then for RT-PCR using serial dilutions of synthetic transcripts. A
25 multiplex assay will be considered successful if it detects all target sequences at a sensitivity of 50 copies plasmid DNA per assay and 100 copies RNA per assay. Successful multiplex assay performance will also include detection of all permutative combinations of two
30 agents to ensure the feasibility of diagnosing simultaneous infection.

Optimize multiplex assay using cell culture extracts

After establishing performance parameters with calibrated
5 synthetic reagents, cell culture extracts of authentic
pathogens will be used. We will recommend specific kits
for nucleic acid extraction and RT-PCR. Nonetheless, we
recognize that some investigators may choose to use other
reagents. Thus, we will assess performance of assays with
10 RNA extracted using readily available commercial systems
that do or do not include organic solvents (e.g. Tri-
Reagent vs RNeasy). Our current protocol employs Tri-
Reagent. Similarly, although we use Superscript reverse
transcriptase (Invitrogen) and HotStart polymerase
15 (QIAGEN), we will also assess the performance of
ThermoScript RT (Invitrogen) at elevated temperature, and
of single-step RT-PCR systems like the Access Kit
(Promega). To optimize efficiency where clinical material
mass is limited and to reduce the complexity of sample
20 preparation, both viral and bacterial agents will be
identified using RT-PCR. In the event network
collaborators agree an agent is characterized by
substantive phylogenetic diversity, cell culture systems
will include at least three divergent isolates of each
25 pathogen. Nasal swabs, sputum and lavage specimens will
be spiked with culture material to optimize recovery
methods for viral as well as bacterial agents. Assays are
validated using banked specimens from naturally infected
humans, and naturally infected animals.

References for Example 5

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Example 6

5 *Primer design and synthesis, template design and synthesis*

Respiratory Panel includes 27 gene targets with validated primer sets as shown below in Table 5.

10 Forward and reverse primer pairs (SEQ ID NOs:1-54) are given for each pathogen (reading from top to bottom starting with RSV-A and ending with C. Pneumoniae). For example, forward primer for RSV-A is SEQ ID NO:1, reverse primer for RSV-A is SEQ ID NO:2. Forward primer

15 for RSV-B is SEQ ID NO:3, reverse primer for RSV-B is SEQ ID NO:4, etcetera.

Table 5: Respiratory Panel Mass-Tag Primers				
Pathogen	Forward primer	Sequence	Reverse primer	Sequence
RSV A	RSA-U1137	AgATCAACTTCTgTCATCCAgC AA	RSV-L1192	gCACATCATAATTAggAgTATCAAT
RSV B	RSB-U1248	AAgATgCAAATCATAAATTCAC AggA	RSV-1318	TgATATCCAgCATCTTTAAgTATCT TTATAgTg
Influenza A (N1)	NA1-U1078	ATggTAATggTgTTTggATAggA Ag	NA1-L1352	AATgCTgCTCCCACTAgTCCAg
Influenza A (N2)	NA2-U560	AAgCATggCTgCATgTTTgTg	NA2-L858	ACCAggATATCgAggATAACAggA
Influenza A (M)	AM-U151	CATggAATggCTAAAgACAAGA CC	AM-L397	AAGTgCACCAgCAGAACTAgAg
Influenza A (H1)	HA1-U583	ggTgTTCATCACCCgTCTAACA T	HA1-L895	gTgTTTgACACTTCgCgTCACAT
Influenza A (H2)	H2A208U27	gCTATgCAAATAAACggAATY CCTCC	H2A559L26	TATTgTTgTACgATCCTTTgCAAC C
Influenza A (H3)	HA3-U115	gCTACTgAgCTggTTCAGAgTT C	HA3-L375	gAAgTCTTCATTgATAAACTCCAg
Influenza A (H5)	HA5human- u71	TTACTgTTACACATgCCCAAgA CA	HA5human- L147	AggYTTCACTCCATTTAgATCgCA
Influenza B	BHA-U188	AgACCAgAggggAAACTATgCCC	BHA-L347	CTgTCgTgCATTATAggAAAgCAC
SARS-CoV	CIID-28891F	AAgCCTCgCCAAAAACgTAC	CIID- 29100R	AAgTCAgCCATgTTCCCGAA
229E-CoV	Taq-Co22- 418F	ggCgCAAgAATTCAGAACCA	Taq-Co22- 636R	TAAgAgCCgCAGCAACTgC
OC43-CoV	Taq-Co43- 270F	TgTgCCTATTgCACCAggAgT	Taq-Co43- 508R	CCCgATCgACAATgTCAGC
Metapneumovirus European	MPV01.2	AACCgTgTACTAAgTgATgCAC TC	MPV02.2	CATTgTTTgACCggCCCCATAA
Metapneumovirus Canadian	MV-Can-U918	AAgTCCAAAggCAGgRCTgTTA TC	MV-Can- L992	CCTgAAgCATTGCCAAgAACACA C
Parainfluenza 1	HPIV1-U82	TACTTTTgACACATTTAgTTCC AggAg	HPIV1-L167	CggTACTTCTTTgACCAggTATAAT Tg
Parainfluenza 2	HPIV2-U908	ggACTTgAACAAgATggCCT	HPIV2-L984	AgCATgAgAgCYTTTAATTTCTggA
Parainfluenza 3	HPIV3-U590	gCTTTCAGACAAGATggAACAg Tg	HPIV3-L668	gCATKATTgACCCAATCTgATCC
Parainfluenza 4A	HPIV4A-U191	AACAgAAggAAATgATggTggAA C	HPIV4A- L269	TgCTgTggATgTATgggCAG
Parainfluenza 4B	HPIV4B-U194	AgAAgAAAACAACgATgAgACA Agg	HPIV4B- L306	gTTTCCCTggTTCACCTCTCTCA
Cytomegalovirus	CMV-U421	TACAgCACgCTCAACACCAAC gCCT	CMV-L501	CCCggCCTTCACCACCAACCgAAA A
Measles virus	MEA-U1103	CAAgCATCATgATgCCATTC CTgg	MEA-L1183	CCTgAATCYCTgCCTATgATgggTT T
Adenovirus	ADV2F-A	CCCMTTYAACCAACCACg	ADV1R-A	ACATCCTTBCKgAAgTTCCA
Enterovirus	5UTR-U447	TCCTCCggCCCCTgAATgCggC TAATCC	5UTR-L541	gAAACACggWCACCCAAAgTASTC g
<i>M. pneumoniae</i>	MTPM1	CCAACCAACAACAACgTTCA	MTPM2	ACCTTgACTggAggCCgTTA
<i>L. pneumophila</i>	Legpneu- U149	gCATWgATgTTARTCCggAAgC A	LegPneu- L223	CggTTAAAgCCAATTgAgCg
<i>C. pneumoniae</i>	CLPM1	CATggTgTCATTgCgCAAgT	CLPM2	CgTgTCgTCCAgCCATTTTA

Table 6, NIAID Priority Agent Panel.

Assays have been designed using 4 primer sets and their cognate synthetic Rift Valley Fever, Crimean Congo Hemorrhagic Fever, Ebola Zaire and Marburg virus

templates created via PCR using overlapping polynucleotides, as shown in Table 6. Forward and reverse primer pairs (SEQ ID NOs:55-62) are given for four of the listed pathogens (reading from top to bottom starting with Rift Valley Fever virus and ending with Marburg virus). For example, forward primer for Rift Valley Fever virus is SEQ ID NO:55, reverse primer for Rift Valley Fever virus is SEQ ID NO:56. Forward primer for CCHF virus is SEQ ID NO:57, reverse primer for CCHF virus is SEQ ID NO:58, etcetera.

Table 6: NIAID Priority Agents Panel Mass-Tag Primers				
Pathogen	Forward primer	Sequence	Reverse primer	Sequence
B. anthracis				
Dengue viruses				
West Nile virus				
Japanese enc. virus				
St. Louis enc. virus				
Yellow Fever virus				
La Crosse virus				
California enc. virus				
Rift Valley Fever virus	RVF-L660	ggATTgACCTgTgCCTgTTgC	RVF-L660	gCATTgAAATgTCCTCTTTgCTgC
CCHF virus	CCHV-L120	AgAACACgTgCCgCTTACgCCCA	CCHV-L120	CCATTCTTYTTTAACTCYTCAAACCA
VEE virus				
EEE virus				
WEE virus				
Ebola virus	EboZA-L319	AACACCGggTCTTAATTCTTATATCAA	EboZA-L319	ggTggTAAAAATCCCATAgTAgTTCTTT
Marburg virus	Mar-L372	TTCCgTCACAAgCCgAAAT T	Mar-L372	TTATTTTAgTTgAgAAAAgAggTTCATgC
LCMV				
Junin virus				
Machupo virus				
Variola virus				

Encephalitis Agent Panel

Table 7 shows primer sets for encephalitis-inducing agents. Forward and reverse primer pairs (SEQ ID NOs:63-96) are given for each pathogen (reading from

- top to bottom starting with West Nile virus and ending with Enterovirus). For example, forward primer for West Nile virus is SEQ ID NO:63, reverse primer for West Nile virus is SEQ ID NO:64. Forward primer for St. Louis Encephalitis virus is SEQ ID NO:65, reverse primer for St. Louis Encephalitis virus is SEQ ID NO:66, etcetera.

Table 7: Encephalitis Agent Panel Mass-Tag Primers				
Pathogen	Forward primer	Sequence	Reverse primer	Sequence
West Nile virus	DF3 -87F	gCTCCgCTgTCCCTgTgA	DF3 -156R	CACTCTCCTCCTgCATggATg
St. Louis enc. virus	SLE-D-73F	CATTgTTCAGCTgTCCAgTC	SLE-D-145R	CTCACCCCTCCCATgAATTgAC
Herpes Simplex virus	HSV-U27	CCCggATgCggTCCAgACgATTAT	HSV-L121	CCCgCggAggTTgTACAAAAAgCT
HIV 1	SK68i	TTCTTlggAgCAgClggAAgCACIATgg	SK69i	TTMATgCCCCAgACIgTIAgTTICAACA
HIV 2	HIV2TMF PR2	ggCTgCACgCCCTATgATA	HIV2TMR PR2	TCTgCATggCTgCTTgATg
N. meningitidis	Nmen-U829	TCTgAAgCCATTggCCgT	Nmen-L892	CAAACACACCACgCgCAT
S. pneumoniae	SPPLY-U532	AgCgATAgCTTCTCCAAGTgg	SPPLY-L606	CTTAGCCAACAAATCgTTTACCg
H. influenzae	HINF-U82	AAgCTCCTTgMATTITTTgTATTAgAA	Hinf-L158	gCTgAATTggCTTgATACCgAg
Influenza B	BHA-U188	AgACCAGAgggAAACTATgCCC	BHA-L347	CTgTCgTgCATTATAggAAAgCAC
SARS-CoV	CIID-28891F	AAgCCTCgCCAAAAACgTAC	CIID-29100R	AAGTCAGCCATgTCCCgAA
229E-CoV	Taq-Co22-418F	ggCgCAAgAATTCAGAACCA	Taq-Co22-636R	TAAgAgCCgCAGCAACTgC
OC43-CoV	Taq-Co43-270F	TgTgCCTATTgCACCAggAgT	Taq-Co43-508R	CCCgATCgACAATgTCAgC
Cytomegalovirus	CMV-U421	TACAgCACgCTCAACACCAACgCCT	CMV-L501	CCCggCCTTACCACCAACCgAAAA
Varicella Zoster virus	VZV-U138	ACgTggATCgTCggATCAGTTgT	VZV-L196	TCgCTATgTgCTAAAACACgCgg
Measles virus	MEA-U1103	CAAgCATCATgATygCCATTCC Tgg	MEA-L1183	CCTgAATCYCTgCCTATgATggTTT
Adenovirus	ADV2F-A	CCCMTTYAACCAACCACg	ADV1R-A	ACATCCTTBCKgAAgTTCCA
Enterovirus	5UTR-U447	TCCTCCggCCCCTgAATgCggCTAATCC	5UTR-L541	gAAACACggWCACCCAAAgTASTCg

10

Improvements in Multiplexing

- Initially, multiplex detection of 7 respiratory pathogen targets at 500 copy sensitivity: RSV group A, RSV group B, Influenza A, HCoV-SARS, HCoV-229E, HCoV-OC43, and *M. pneumoniae* was determined. Subsequently, sensitivity was improved. Detection at 100 copy

sensitivity has been confirmed for 18 respiratory pathogen targets in a 20-plex assay (Table 8). Two of 20 targets, the influenza A M gene and influenza H1 gene, were detected at 500 copies. This typically
5 corresponds in our laboratory to <0.001 TCID₅₀ per assay, a threshold comparable to many useful microbiological assays.

Table 8: Sensitivity of respiratory panel

	RSV A	RSV B	Influenza A (N1)	Influenza A (N2)	Influenza A (matrix)	Influenza A (H1)	Influenza A (H2)	Influenza A (H3)	Influenza A (H5)	Influenza B
500 copies	+	+	+	+	+	+	+	+	+	+
100 copies	+	+	+	+	-	-	+	+	+	+

	HCoV- SARS	HCoV- 229E	HCoV- OC43	Meta-pneu- mo-virus (Eur.)	HPIV -1	HPIV- 2	HPIV-3	<i>M.</i> <i>pneumoniae</i>	<i>C.</i> <i>pneumoniae</i>	<i>L.</i> <i>pneumophila</i>
500 copies	+	+	+	+	+	+	+	+	+	+
100 copies	+	+	+	+	+	+	+	+	+	+

Clinical Samples

5 Although assays of synthetic targets were optimized in
 a complex background of normal tissue nucleic acids,
 analysis of clinical materials was performed. Banked
 clinical respiratory specimens were obtained from
 Cinnia Huang of the Wadsworth Laboratory of the New
 10 York State Department of Health and Pilar Perez-Brena
 of the National Center for Microbiology of Spain.
 Organisms included: metapneumovirus (n=3), RSV-B (n=3),
 RSV-A (n=2), adenovirus (n=2), HPIV-1 (n=1), HPIV-3
 (n=2), HPIV-4 (n=2), enterovirus (n=2), SARS-CoV (n=4),
 15 influenza A (n=2). Six representative results are shown
 in Figure 18; Multiplex Mass Tag PCR analysis of six
 human respiratory specimens. Signal to noise ratio is
 on the ordinate and primer sets are listed on the
 abscissa. Mass Tag primer sets employed in a single
 20 tube assay are indicated at the bottom of the figure.
 Fig. 18A - Influenza A (N1, M, H1) H1); 18B - Human
 Parainfluenza Type 1; 18C - Respiratory Syncytial Group
 B; 18D - Enterovirus; 18E - SARS CoV; and 18F - Human
 Parainfluenza Type 3.

25 *Pathogens*

Tables 9-12 show a non-comprehensive list of various
 target pathogens and corresponding primer sequences. In
 30 Table 10, the forward and reverse primer pairs for
 Cytomegalovirus, SEQ ID NOS: 87 and 88; for HPIV-4A,
 SEQ ID NOS: 37 and 38; for HPIV-4B, SEQ ID NOS: 39 and
 40; for Measles, SEQ ID NOS: 91 and 92; for Varicella
 Zoster virus, SEQ ID NOS: 89 and 90; for HIV-1, SEQ ID
 35 NOS: 69 and 70; for HIV-2, SEQ ID NOS: 71 and 72; for
 S. Pneumoniae, SEQ ID NOS: 100 and 101; for Haemophilus

Influenzae, SEQ ID NOS: 77 and 78; for Herpes Simplex, SEQ ID NOS: 67 and 68; for MV Canadian isolates, SEQ ID NOS: 29 and 30; for Adenovirus 2 A/B 505/630, SEQ ID NOS: 93 and 94; for Enterovirus A/B 702/495, SEQ ID NOS: 95 and 96; and forward primers for Enterovirus A/B 702/495, SEQ ID NOS: 98 and 99.

10.

Table 9

Primer sequence	Name	Target	Previous Masscode	Panel
HIV2	HIV2TMFPR2	Forward A	588	Respiratory / Enc
HIV2	HIV2TMRRP2	Reverse B	570	Respiratory / Enc
Streptococcus pneumoniae	SPPLY-U532	Forward A	714	Respiratory / Enc
Streptococcus pneumoniae	SPPLY-L606	Reverse B	694	Respiratory / Enc
Haemophilus influenza	HINF-U82	Forward A	734	Respiratory / Enc
Haemophilus influenza	Hinf-L158	Reverse B	726	Respiratory / Enc
Herpes Simplex	HSV-U27	Forward A	722	Respiratory / Enc
Herpes Simplex	HSV-L121	Reverse B	708	Respiratory / Enc
Metaneumovirus Canadian	MV-Can-U918	Forward A	718	Respiratory
Metaneumovirus Canadian	MV-Can-L992	Reverse B	654	Respiratory
Adenovirus	ADV2F-A	Forward A	503	Respiratory / Enc
Adenovirus	ADV1R-A	Reverse B	630	Respiratory / Enc
Enterovirus	5UTR-U447	Forward A	702	Respiratory / Enc
Enterovirus	5UTR-U450	Forward A	702	Respiratory / Enc
Enterovirus	5UTR-u457	Forward A	702	Respiratory / Enc
Enterovirus	5UTR-L541	Reverse B	495	Respiratory / Enc
Neisseria meningitidis	Nmen-U829	Forward A	730	Encephalitis / Resp
Neisseria meningitidis	Nmen-L892	Reverse B	439	Encephalitis / Resp
WNV1	DF3-87F	Forward A	539	Encephalitis
WNV1	DF3-156R	Reverse B	499	Encephalitis
WNV2	WN-Ax-FWD	Forward A	539	Encephalitis
WNV2	WN-Ax-REV	Reverse B	499	Encephalitis
SLE	SLE-D-73F	Forward A	658	Encephalitis
SLE	SLE-D-145R	Reverse B	642	Encephalitis

Table 9. (Cont.)

Primer sequence	Name	Target	Previous Masscode	Panel
Cytomegalovirus	CMV-U421	Forward A	620	Respiratory / Enc
Cytomegalovirus	CMV-L501	Reverse B	610	Respiratory / Enc
HPV4A	HPV4A-U191	Forward A	622	Respiratory
HPV4A	HPV4A-L269	Reverse B	609	Respiratory
HPV4B	HPV4B-U194	Forward A	622	Respiratory
HPV4B	HPV4B-L306	Reverse B	606	Respiratory
Measles	MEA-U1103	Forward A	678	Respiratory / Enc
Measles	MEA-L1183	Reverse B	662	Respiratory / Enc
VZV	VZV-U138	Forward A	615	Respiratory / Enc
VZV	VZV-L196	Reverse B	671	Respiratory / Enc
HIV1	SK681		674	Respiratory / Enc
HIV1	SK691		383	Respiratory / Enc

Table 9 (Cont.)

Primer sequence	Name	Target	Previous Masscode	Panel
RSV A gen N	RS-A-U1137	Forward A	467	Respiratory
RSV A gen N	RSV-L1192	Reverse B	455	Respiratory
RSV B gen N	RSB-U1248	Forward A	483	Respiratory
RSV B gen N	RSV-1318	Reverse B	479	Respiratory
Flu A - N1	NA1-U1078	Forward A	489	Respiratory
Flu A - N1	NA1-L1352	Reverse B	439	Respiratory
Flu A - N2	NA2-U560	Forward A	658	Respiratory
Flu A - N2	NA2-L858	Reverse B	730	Respiratory
Flu A (MATRIX)	AM-U151	Forward A	618	Respiratory / Enc
Flu A (MATRIX)	AM-L397	Reverse B	690	Respiratory / Enc
Flu B	BHA-U188	Forward A	688	Respiratory / Enc
Flu B	BHA-L347	Reverse B	598	Respiratory / Enc
SARS-Coronavirus	CIID-28891F	Forward A	527	Respiratory / Enc
SARS-Coronavirus	CIID-29100R	Reverse B	666	Respiratory / Enc
229E-Coronavirus	Taq-Co22-418F	Forward A	670	Respiratory / Enc
229E-Coronavirus	Taq-Co22-638R	Reverse B	558	Respiratory / Enc
OC43-Coronavirus	Taq-Co43-270F	Forward A	686	Respiratory / Enc
OC43-Coronavirus	Taq-Co43-508R	Reverse B	548	Respiratory / Enc
Metapneumovirus	MPV01.2	Forward A	718	Respiratory
Metapneumovirus	MPV02.2	Reverse B	654	Respiratory
Mycoplasma pneumoniae	MTPM1	Forward A	602	Respiratory
Mycoplasma pneumoniae	MTPM2	Reverse B	614	Respiratory

Table 9 (Cont.)

Primer sequence	Name	Target	Previous Masscode	Panel
adenovirus	ADV1F-A	Forward A	503	Respiratory / Enc
adenovirus	ADV2R-A	Reverse B	630	Respiratory / Enc
Chlamydia	CLPM1	Forward A	519	Respiratory
Chlamydia	CLPM2	Reverse B	571	Respiratory
enterovirus	EV1F	Forward A	702	Respiratory / Enc
enterovirus	EV1R	Reverse B	485	Respiratory / Enc
flavivirus1	Fla-U8093	Forward A	710	Encephalitis
flavivirus1	Fla-L9279	Reverse B	594	Encephalitis
flavivirus2	Fla-U8954	Forward A	710	Encephalitis
flavivirus2	Fla-L10098	Reverse B	594	Encephalitis
fluhA1	HA1-U583	Forward A	650	Respiratory
fluhA1	HA1-L895	Reverse B	634	Respiratory
fluhA2	H2A208U27	Forward A	662	Respiratory
fluhA2	H2A559L26	Reverse B	638	Respiratory
fluhA3	HA3-U115	Forward A	375	Respiratory
fluhA3	HA3-L380	Reverse B	475	Respiratory
fluhA5	HA5-U71	Forward A	646	Respiratory
fluhA5	HA5-L147	Reverse B	395	Respiratory
HPV1	HPV1-U82	Forward A	566	Respiratory
HPV1	HPV1-L167	Reverse B	357	Respiratory
HPV2	HPV2-U808	Forward A	483	Respiratory
HPV2	HPV2-L984	Reverse B	590	Respiratory
HPV3	HPV3-U590	Forward A	642	Respiratory
HPV3	HPV3-L668	Reverse B	539	Respiratory
Legionella1	LegPneu-U149	Forward A	678	Respiratory
Legionella1	LegPneu-L223	Reverse B	582	Respiratory

Respiratory Panel Mass-Tag Primers

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Table 11

Tagged Pairs	Standards	LIST OF PRIMERS	Name FWD	Forward - A	Im primer	Name REV	Reverse - B	Im primer	Product Size
RSVA - 1 AB 467455	YES	RSVA pen N	RSVA-U137	AGATCACTCTCTGTCATCCAGCA	62	RSVA-L182	GGCATCATTAATAGGATATCAAT	56	60
RSVB - 1 AB 483479	YES	RSVB pen N	RSVB-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVB-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	320
RSVA pen N	YES	RSVA pen P	RSVA-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVA-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	105
RSVB pen N	YES	RSVB pen P	RSVB-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVB-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	180
RSVA pen N	YES	RSVA pen P	RSVA-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVA-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	120
RSVB pen N	YES	RSVB pen P	RSVB-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVB-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	214
RSVA pen N	YES	RSVA pen P	RSVA-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVA-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	268
RSVB pen N	YES	RSVB pen P	RSVB-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVB-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	246
RSVA pen N	YES	RSVA pen P	RSVA-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVA-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	159
RSVB pen N	YES	RSVB pen P	RSVB-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVB-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	130
RSVA pen N	YES	RSVA pen P	RSVA-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVA-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	240
RSVB pen N	YES	RSVB pen P	RSVB-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVB-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	260
RSVA pen N	YES	RSVA pen P	RSVA-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVA-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	205
RSVB pen N	YES	RSVB pen P	RSVB-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVB-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	76
RSVA pen N	YES	RSVA pen P	RSVA-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVA-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	380
RSVB pen N	YES	RSVB pen P	RSVB-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVB-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	110
RSVA pen N	YES	RSVA pen P	RSVA-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVA-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	102
RSVB pen N	YES	RSVB pen P	RSVB-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVB-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	103
RSVA pen N	YES	RSVA pen P	RSVA-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVA-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	79
RSVB pen N	YES	RSVB pen P	RSVB-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVB-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	75
RSVA pen N	YES	RSVA pen P	RSVA-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVA-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	100
RSVB pen N	YES	RSVB pen P	RSVB-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVB-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	85
RSVA pen N	YES	RSVA pen P	RSVA-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVA-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	312
RSVB pen N	YES	RSVB pen P	RSVB-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVB-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	377
RSVA pen N	YES	RSVA pen P	RSVA-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVA-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	260
RSVB pen N	YES	RSVB pen P	RSVB-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVB-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	265
RSVA pen N	YES	RSVA pen P	RSVA-U1248	ATGCTTCCAGGCAATGATGCT	62	RSVA-L1318	TGATATCCAGCATTTAATGATTTATGTG	63	105

Table 12

Primer sequence	Name	Target	Previous Message	Panel
TACAGCAGGCTCAACACCAACGCCT	25 CMV-U421	Citomegalovirus		Respiratory
AACAGAAAGGAATGATGGTGAAC	24 HPV4A-U191	HPV4A		Respiratory
AGAAGAAACAAACGATGAGCAAGG	25 HPV4B-U194	HPV4B		Respiratory
CAAGCATCATGATGCGCATTCCTGG	25 MEA-U1103	Measles		Respiratory
ACGTGGATCGTGGATCAGTTGT	23 VZV-U138	VZV		Respiratory
TTCTTGGAGCAGCAGGAGGACATGG	28 SK68i	HIV1		Respiratory
GGCTGCACGCCCTATGATA	19 HIV2TMFPR2	HIV2		Respiratory
AGCGATAGCTTTCTCCAATGG	22 SPPLY-U532	<i>Streptococcus pneumoniae</i>		Respiratory
AAGCTCCCTGMAATTTTTTGTATTAGAA	27 HINF-U82	<i>Haemophilus influenzae</i>		Respiratory
CCCGGATGCCGTCCAGACGATTAT	24 HSV-U27	Herpes Simplex		Respiratory
AAGTCCAAAGGCAGGCTGTTATC	24 MV-Can-U918	Metanemovirus Canadian		Respiratory
CGCMTTYAACCAACCCG	18 ADV2F-A	Adenovirus	Adenovirus2 503	Respiratory
TCCTCCGGCCCTGAATGCGGCTAATCC	28 SUTR-U447	Enterovirus	Enterovirus 702	Respiratory
TCGGCCCTGGAATGCGGCTAATCC	25 SUTR-U450	Enterovirus	Enterovirus 702	Respiratory
CCCTGAAATGCCGCTAATCC	20 SUTR-U457	Enterovirus	Enterovirus 702	Respiratory
CCCGGCCCTCAGCACCAACCGAAAA	25 CMV-L501	Citomegalovirus		Respiratory
TGCTGTGGATGTATGGGCG	20 HPV4A-L269	HPV4a		Respiratory
GTTTCCCTGGTCACTCTCTTCA	23 HPV4B-L306	HPV4b		Respiratory
CCTGAATCYCTGCCATGATGGGTTT	28 MEA-L1183	Measles		Respiratory
TCGCTATGTGCTAAACACGCGG	23 VZV-L198	VZV		Respiratory
ITMATGCCCCAGACGCTGATTTCAACA	28 SK69i	HIV1		Respiratory
ICTGATGCGTGTGATG	19 HIV2TMFPR2	HIV2		Respiratory
GTTAGCCAAACATCGTTTACCG	23 SPPLY-L606	<i>Streptococcus pneumoniae</i>		Respiratory
GCTGAATGGCTTGTGATACCGAG	23 Hinf-L158	<i>Haemophilus influenzae</i>		Respiratory
CCCGCGGAGGTGTGACAAAAAGCT	24 HSV-L121	Herpes Simplex		Respiratory
CCTGAAGCATTTCCCAAGAACACAC	25 MV-Can-L992	Metanemovirus Canadian		Respiratory
ACATCCTTBCXGAAGTTCCA	20 ADV1R-A	Adenovirus	Adenovirus2 630	Respiratory
GAAACACGGWCACCCAAAGTASTCG	25 SUTR-L541	Enterovirus	Enterovirus 495	Respiratory
AACACCGGGTCTTAATTTCTATATCAA	27 Ebo2a-U234	Ebola Zaire		Hemorrhagic Fevers
TTCCGTCACAAGCCGAATTT	20 Mar-U292	Marburg		Hemorrhagic Fevers
AGAACACGTGGCGCTTACGCCCA	23 CCHV-U44	CCHV		Hemorrhagic Fevers
TCCCAAGATGTTAGTGCGTGA	22 Sabia-U344	Sabia		Hemorrhagic Fevers
CCACCGGTACCTGAGACACAAATT	26 Machupo-U212	Machupo		Hemorrhagic Fevers
GCTGGGAGCGCGGTATC	17 RVF-U185	Yellow Fever		Hemorrhagic Fevers
CGATTGACCTGTGGCTGTTC	17 RVF-U578	Rift Valley fever		Hemorrhagic Fevers
TCTGAAGCATTTGGCGCT	18 Nmen-U829	<i>Neisseria meningitidis</i>		Hemorrhagic Fevers
CTATTATTANTGGCTATAAGTTTGC	27 RSF-U233	<i>Rickettsia spotted fever</i>		Hemorrhagic Fevers
VACATGACMGATGAGGTTGTRGC	24 Bburg-U898	Borrelia burgdorferi		Hemorrhagic Fevers
GATGGAGGRTGCATCATGG	19 OMSK-U171	OMSK		Hemorrhagic Fevers
AACCTAGGAGGTACCCAAACACGC	24 CHKP-U68	Chikungunya POL		Hemorrhagic Fevers
CAATGTCTTCMCGCTGGACACCT	23 CHKE-U223	Chikungunya ENV		Hemorrhagic Fevers
AYACAGCAGCAGTTAGGCTCTCT	22 HAN-U179	Hanlun		Hemorrhagic Fevers
ATGAARGCAGATGARATYACAC	23 DOB-U222	Dobrava		Hemorrhagic Fevers
AAGGTGTTTTTATCAGGCTAGAGA	25 TAC-U114	Tacaribe		Hemorrhagic Fevers
GCCRTGTGARTGCCRTCTTCCATT	24 GUAV-U321	Guanarito		Hemorrhagic Fevers
CAGGATTCGACAGGCGGAAGA	20 SEC-U243	Seoul		Hemorrhagic Fevers
TGGAAGCCTCGCTCAAGAG	20 KYF-U170	Kyasanur forest		Hemorrhagic Fevers
TGACCTTYACMAATGATTCAT	22 LCMV-U478	LCMV		Hemorrhagic Fevers
GCTGGTAAATTCCTATAGTATCTTT	28 Ebo2a-L319	Ebola Zaire		Hemorrhagic Fevers
TTATTTTATGTTGAGAAAAGAGTTTCATGC	29 Mar-L372	Marburg		Hemorrhagic Fevers
CCATTCTTTTTRAACTCTTCAACCA	27 CCHV-L120	CCHV		Hemorrhagic Fevers
CCTGCACTGACAATCGCTTG	20 SABIA-L424	Sabia		Hemorrhagic Fevers
TGCAAGTCAAGCGAAAGAGGGGATG	26 Machupo-L290	Machupo		Hemorrhagic Fevers
GGAAGCCCAATGGTCTCAT	20 YF-L249	Yellow Fever		Hemorrhagic Fevers
GCATTAGAAATGTCTCTTTTGTGTC	26 RVF-L660	Rift Valley fever		Hemorrhagic Fevers
CAACACACCCAGCGCAT	18 Nmen-L892	<i>Neisseria meningitidis</i>		Hemorrhagic Fevers
ACRRTTTAAAGTTAARCTTTTGCC	24 RSF-L394	<i>Rickettsia spotted fever</i>		Hemorrhagic Fevers
GCAATGACAAAACATATTGCGGAATTGA	29 Bburg-L877	Borrelia burgdorferi		Hemorrhagic Fevers
TGACCACTTGGCTGATCC	19 OMSK-L234	OMSK		Hemorrhagic Fevers
GGACGGTACAGCGCTTCTG	19 CHKP-L132	Chikungunya POL		Hemorrhagic Fevers
TCRCCAAATGTGCTGGTCTTCTGT	25 CHKE-L310	Chikungunya ENV		Hemorrhagic Fevers
GCTGGCTGAGTGTGCTGCTT	22 HAN-L245	Hanlun		Hemorrhagic Fevers
CCTGCGCTGGATATATCCACA	22 DOB-L289	Dobrava		Hemorrhagic Fevers
CCATCTTATGTTGTTAATCATG	23 TAC-L192	Tacaribe		Hemorrhagic Fevers
TATGTRCACTGTTTCAGAAAACCTCA	26 GUA-L265	Guanarito		Hemorrhagic Fevers
ATGATCACCAGGTTTCTACCC	21 SECUL-L309	Seoul		Hemorrhagic Fevers
TCATCCCACTGACCGCAT	20 KYF-L233	Kyasanur forest		Hemorrhagic Fevers
TATRCTCATGAGTGTGTGGTCAA	23 LCMV-L142a	LCMV	Same than below	Hemorrhagic Fevers
TATRCTCATAGTGTGTGATCAA	23 LCMV-L142b	LCMV	Same than above	Hemorrhagic Fevers

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Example 7

Efficient laboratory diagnosis of infectious diseases is increasingly important to clinical management and public health. Methods to directly detect nucleic acids of microbial pathogens in clinical specimens are rapid, sensitive, and may succeed when culturing the organism fails. Clinical syndromes are infrequently specific for single pathogens; thus, assays are needed that allow multiple agents to be simultaneously considered. Current multiplex assays employ gel-based formats in which products are distinguished by size, fluorescent reporter dyes that vary in color, or secondary enzyme hybridization assays. Gel-based assays are reported that detect 2-8 different targets with sensitivities of 2-100 PFU or less than 1-5 PFU, depending on whether amplification is carried out in a single or nested format, respectively (1-4). Fluorescence reporter systems achieve quantitative detection with sensitivity similar to that of nested amplification; however, their capacity to simultaneously query multiple targets is limited to the number of fluorescent emission peaks that can be unequivocally resolved. At present, up to 4 fluorescent reporter dyes can be detected simultaneously (5,6). Multiplex detection of up to 9 pathogens has been achieved in hybridization enzyme systems; however, the method requires cumbersome postamplification processing (7).

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Experimental Results

To address the need for sensitive multiplex assays in diagnostic molecular microbiology, we created a polymerase chain reaction (PCR) platform in which microbial gene targets are coded by a library of 64 distinct Masscode tags (Qiagen Masscode technology, Qiagen, Hilden, Germany). A schematic representation of this approach is shown in Figure 22. Microbial nucleic acids (RNA, DNA, or both) are amplified by multiplex reverse transcription (RT)-PCR using primers labeled by a photocleavable link to molecular tags of different molecular weight. After removing unincorporated primers, tags are released by UV irradiation and analyzed by mass spectrometry. The identity of the microbe in the clinical sample is determined by its cognate tags. As a first test of this technology, we focused on respiratory disease because differential diagnosis is a common clinical challenge, with implications for outbreak control and individual case management. Multiplex primer sets were designed to identify up to 22 respiratory pathogens in a single Mass Tag PCR reaction; sensitivity was established by using synthetic DNA and RNA standards as well as titered viral stocks; the utility of Mass Tag PCR was determined in blinded analysis of previously diagnosed clinical specimens. Oligonucleotide primers were designed in conserved genomic regions to detect the broadest number of members for a given pathogen species by efficiently amplifying a 50- to 300-bp product. In some instances, we selected established primer sets; in others, we used a software program designed to cull sequence information from GenBank, perform multiple

alignments, and maximize multiplex performance by selecting primers with uniform melting temperatures and minimal cross-hybridization potential (Appendix Table, available at http://www.cdc.gov/ncidod/eid/vol11no02/04-0492_app.htm). Primers, synthesized with a 5'C6 spacer and aminohexyl modification, were covalently conjugated by a photocleavable link to Masscode tags (Qiagen Masscode technology) (8,9). Masscode tags have a modular structure, including a tetrafluorophenyl ester for tag conjugation to primary amines; an o-nitrobenzyl photolabile linker for photoredox cleavage of the tag from the analyte; a mass spectrometry sensitivity enhancer, which improves the efficiency of atmospheric pressure chemical ionization of the cleaved tag; and a variable mass unit for variation of the cleaved tag mass (8,10-12). A library of 64 different tags has been established. Forward and reverse primers in individual primer sets are labeled with distinct molecular weight tags. Thus, amplification of a microbial gene target produces a dual signal that allows assessment of specificity. Gene target standards were cloned by PCR into pCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA) by using DNA template (bacterial and DNA viral targets) or cDNA template (RNA viral targets) obtained by reverse transcription of extracts from infected cultured cells or by assembly of overlapping synthetic polynucleotides. Assays were initially established by using plasmid standards diluted in 2.5- μ g/mL human placenta DNA (Sigma, St. Louis, MO, USA) and subjected to PCR amplification with a multiplex PCR kit (Qiagen), primers at 0.5 μ mol/L each, and the following cycling protocol:

an annealing step with a temperature reduction in 1°C increments from 65°C to 51°C during the first 15 cycles and then continuing with a cycling profile of 94°C for 20 s, 50°C for 20 s, and 72°C for 30 s in an MJ PTC200 thermal cycler (MJ Research, Waltham, MA, USA). Amplification products were separated from unused primers by using QIAquick 96 PCR purification cartridges (Qiagen, with modified binding and wash buffers). Masscode tags were decoupled from amplified products through UV light-induced photolysis in a flow cell and analyzed in a single quadrupole mass spectrometer using positive-mode atmospheric pressure chemical ionization (Agilent Technologies, Palo Alto, CA, USA). A detection threshold of 100 DNA copies was determined for 19 of 22 cloned targets by using a 22-plex assay (Table 1). Many respiratory pathogens have RNA genomes; thus, where indicated, assay sensitivity was determined by using synthetic RNA standards or RNA extracts of viral stocks. Synthetic RNA standards were generated by using T7 polymerase and linearized plasmid DNA. After quantitation by UV spectrometry, RNA was serially diluted in 2.5-μg/mL yeast tRNA (Sigma), reverse transcribed with random hexamers by using Superscript II (Invitrogen, Carlsbad, CA, USA), and used as template for Mass Tag PCR. As anticipated, sensitivity was reduced by the use of RNA instead of DNA templates (Table 15).

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Table 15

Pathogen or protein	Detection threshold (DNA copies/RNA copies)
Influenza A matrix	100/1,000
Influenza A N1	100/NA
Influenza A N2	100/NA
Influenza A H1	100/NA
Influenza A H2	100/NA
Influenza A H3	100/NA
Influenza A H5	100/NA
Influenza B H	500/1,000
RSV group A	100/1,000
RSV group B	100/500
Metapneumovirus	100/1,000
CoV-SARS	100/500
CoV-OC43	100/500
CoV-229E	100/500
HPIV-1	100/1,000
HPIV-2	100/1,000
HPIV-3	100/500
<i>Chlamydia pneumoniae</i>	100/NA
<i>Mycoplasma pneumoniae</i>	100/NA
<i>Legionella pneumophila</i>	100/NA
Enterovirus (genus)	500/1,000
Adenovirus (genus)	5,000/NA

*NA, not assessed; RSV, respiratory syncytial virus; CoV, coronavirus; SARS, severe acute respiratory syndrome; HPIV, human parainfluenza virus.

5 The sensitivity of Mass Tag PCR to detect live virus was tested by using RNA extracted from serial dilutions of titrated stocks of coronaviruses (severe acute respiratory syndrome [SARS] and OC43) and

0 parainfluenzaviruses (HPIV 2 and 3). A 100- μ L volume of each dilution was analyzed. RNA extracted from a 1-TCID₅₀/mL dilution, representing 0.025 TCID₅₀ per PCR reaction, was consistently positive in Mass Tag PCR. RNA extracted from banked sputum, nasal swabs, and pulmonary

5 washes of persons with respiratory infection was tested by using an assay panel comprising 30 gene targets that

represented 22 respiratory pathogens. Infection in each of these persons had been previously diagnosed through virus isolation, conventional nested RT-PCR, or both. Reverse transcription was performed using random
 5 hexamers, and Mass Tag PCR results were consistent in all cases with the established diagnosis. Infections with respiratory syncytial virus, human parainfluenza virus, SARS coronavirus, adenovirus, enterovirus, metapneumovirus, and influenza virus were correctly
 0 identified (Table 16 and Figure 23).

Table 16

Pathogen	No. positive/no. tested†
RSV A	2/2
RSV B	3/3
HPIV-1	1/1
HPIV-3	2/2
HPIV-4	2/2
CoV-SARS	4/4
Metapneumovirus	2/3
Influenza B	1/3
Influenza A	2/6
Adenovirus	2/2
Enterovirus	2/2

*RSV, respiratory syncytial virus; HPIV, human parainfluenza virus; CoV, coronavirus; SARS, severe acute respiratory syndrome.
 †No. positive and consistent with previous diagnosis/number tested (with respective previous diagnosis).

5

A panel comprising gene targets representing 17 pathogens related to central nervous system infectious
 0 disease (influenza A virus matrix gene; influenza B virus; human coronaviruses 229E, OC43, and SARS; enterovirus; adenovirus; human herpesvirus-1 and -3; West Nile virus; St. Louis encephalitis virus; measles virus; HIV-1 and -2; and *Streptococcus pneumoniae*,

Haemophilus influenzae, and *Nisseria meningitidis*) was applied to RNA obtained from banked samples of cerebrospinal fluid and brain tissue that had been previously characterized by conventional diagnostic RT-PCR. Two of 3 cases of West Nile virus encephalitis were correctly identified. Eleven of 12 cases of enteroviral meningitis were detected representing serotypes CV-B2, CV-B3, CV-B5, E-6, E-11, E-13, E-18, and E-30 (data not shown).

0

Conclusions

Our results indicate that Mass Tag PCR is a sensitive and specific tool for molecular characterization of microflora. The advantage of Mass Tag PCR is its capacity for multiplex analysis. Although the use of degenerate primers (e.g., enteroviruses and adenoviruses, and Table 16) may reduce sensitivity, the limit of multiplexing to detect specific targets will likely be defined by the maximal primer concentration that can be accommodated in a PCR mix. Analysis requires the purification of product from unincorporated primers and mass spectroscopy. Although these steps are now performed manually, and mass spectrometers are not yet widely distributed in clinical laboratories, the increasing popularity of mass spectrometry in biomedical sciences and the advent of smaller, lower-cost instruments could facilitate wider use additional pathogen panels, our continuing work is focused on optimizing multiplexing, sensitivity, and throughput. Potential applications include differential diagnosis of

infectious diseases, blood product surveillance,
forensic microbiology, and biodefense.

What is claimed is:

1. A method for simultaneously detecting in a sample the presence of one or more of a plurality of different target nucleic acids comprising the steps of:
 - (a) contacting the sample with a plurality of nucleic acid primers simultaneously and under conditions permitting, and for a time sufficient for, primer extension to occur, wherein (i) for each target nucleic acid at least one predetermined primer is used which is specific for that target nucleic acid, (ii) each primer has a mass tag of predetermined size bound thereto via a labile bond, and (iii) the mass tag bound to any primer specific for one target nucleic acid has a different mass than the mass tag bound to any primer specific for any other target nucleic acid;
 - (b) separating any unextended primers from any extended primers;
 - (c) simultaneously cleaving the mass tags from any extended primers; and
 - (d) simultaneously determining the presence and sizes of any mass tags so cleaved,wherein the presence of a cleaved mass tag having the same size as a mass tag of predetermined size previously bound to a predetermined primer indicates the presence in the sample of the target nucleic acid specifically recognized by that predetermined primer.

2. The method of claim 1, wherein the method detects the presence in the sample of 10 or more different target nucleic acids.
3. The method of claim 1, wherein the method detects the presence in the sample of 50 or more different target nucleic acids.
4. The method of claim 1, wherein the method detects the presence in the sample of 100 or more different target nucleic acids.
5. The method of claim 1, wherein the method detects the presence in the sample of 200 or more different target nucleic acids.
6. The method of claim 1, wherein the sample is contacted with 4 or more different primers.
7. The method of claim 1, wherein the sample is contacted with 10 or more different primers.
8. The method of claim 1, wherein the sample is contacted with 50 or more different primers.
9. The method of claim 1, wherein the sample is contacted with 100 or more different primers.
10. The method of claim 1, wherein the sample is contacted with 200 or more different primers.

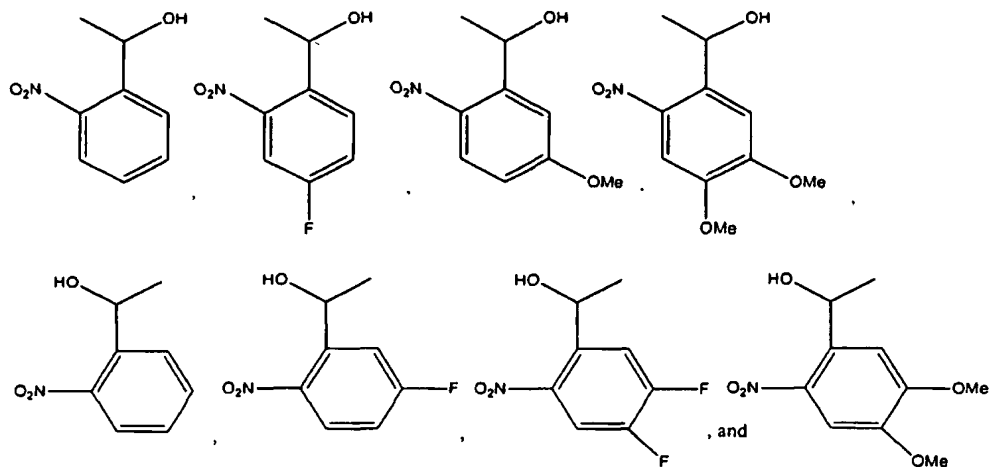
11. The method of claim 1, wherein one or more primers comprises the sequence set forth in one of SEQ ID NOs:1-96.
12. The method of claim 1, wherein at least two different primers are specific for the same target nucleic acid.
13. The method of claim 12, wherein a first primer is a forward primer for the target nucleic acid and a second primer is a reverse primer for the same target nucleic acid.
14. The method of claim 13, wherein the mass tags bound to the first and second primers are of the same size.
15. The method of claim 13, wherein the mass tags bound to the first and second primers are of a different size.
16. The method of claim 12, wherein a first primer is directed to a 5'-UTR of the target nucleic acid and a second primer is directed to a 3D polymerase region of the target nucleic acid.
17. The method of claim 1, wherein each primer is from 15 to 30 nucleotides in length.
18. The method of claim 1, wherein each mass tag has a molecular weight of from 100Da to 2,500Da.

19. The method of claim 1, wherein the labile bond is a photolabile bond.
20. The method of claim 19, wherein the photolabile bond is cleavable by ultraviolet light.
21. The method of claim 1, wherein at least one target nucleic acid is from a pathogen.
22. The method of claim 21, wherein the pathogen is selected from the group consisting of B. anthracis, a Dengue virus, a West Nile virus, Japanese encephalitis virus, St. Louis encephalitis virus, Yellow Fever virus, La Crosse virus, California encephalitis virus, Rift Valley Fever virus, CCHF virus, VEE virus, EEE virus, WEE virus, Ebola virus, Marburg virus, LCMV, Junin virus, Machupo virus, Variola virus, SARS corona virus, an enterovirus, an influenza virus, a parainfluenza virus, a respiratory syncytial virus, a bunyavirus, a flavivirus, and an alphavirus.
23. The method of claim 21, wherein the pathogen is a respiratory pathogen.
24. The method of claim 23, wherein the respiratory pathogen is selected from the group consisting of respiratory syncytial virus A, respiratory syncytial virus B, Influenza A (N1), Influenza A (N2), Influenza A (M), Influenza A (H1), Influenza A (H2), Influenza A (H3), Influenza A (H5), Influenza B, SARS coronavirus, 229E coronavirus, OC43 coronavirus, Metapneumovirus European,

Metapneumovirus Canadian, Parainfluenza 1, Parainfluenza 2, Parainfluenza 3, Parainfluenza 4A, Parainfluenza 4B, Cytomegalovirus, Measles virus, Adenovirus, Enterovirus, M. pneumoniae, L. pneumophila, and C. pneumoniae.

25. The method of claim 21, wherein the pathogen is an encephalitis-inducing pathogen.
26. The method of claim 25, wherein the encephalitis-inducing pathogen is selected from the group consisting of West Nile virus, St. Louis encephalitis virus, Herpes Simplex virus, HIV 1, HIV 2, N. meningitides, S. pneumoniae, H. influenzae, Influenza B, SARS coronavirus, 229E-CoV, OC43-CoV, Cytomegalovirus, and a Varicella Zoster virus.
27. The method of claim 21, wherein the pathogen is a hemorrhagic fever-inducing pathogen.
28. The method of claim 1, wherein the sample is a forensic sample.
29. The method of claim 1, wherein the sample is a food sample.
30. The method of claim 1, wherein the sample is blood, or a derivative of blood.
31. The method of claim 1, wherein the sample is a biological warfare agent or a suspected biological warfare agent.

32. The method of claim 1, wherein the mass tag is selected from the group consisting of:



33. The method of claim 1, wherein the presence and size of any cleaved mass tag is determined by mass spectrometry.
34. The method of claim 33, wherein the mass spectrometry is selected from the group consisting of atmospheric pressure chemical ionization mass spectrometry, electrospray ionization mass

spectrometry, and matrix assisted laser desorption ionization mass spectrometry.

35. The method of claim 1, wherein the target nucleic acid is a ribonucleic acid.
36. The method of claim 1, wherein the target nucleic acid is a deoxyribonucleic acid.
37. The method of claim 1, wherein the target nucleic acid is from a viral source.
38. A kit for simultaneously detecting in a sample the presence of one or more of a plurality of different target nucleic acids comprising a plurality of nucleic acid primers wherein (i) for each target nucleic acid at least one predetermined primer is used which is specific for that target nucleic acid, (ii) each primer has a mass tag of predetermined size bound thereto via a labile bond, and (iii) the mass tag bound to any primer specific for one target nucleic acid has a different mass than the mass tag bound to any primer specific for any other target nucleic acid.
39. A kit for simultaneously detecting in a sample the presence of one or more of a plurality of different target nucleic acids comprising (a) a plurality of nucleic acid primers wherein (i) for each target nucleic acid at least one predetermined primer is used which is specific for that target nucleic acid, (ii) each primer has a mass tag of predetermined size bound thereto via a labile bond, and (iii) the

mass tag bound to any primer specific for one target nucleic acid has a different mass than the mass tag bound to any primer specific for any other target nucleic acid; and (b) a mass spectrometer.

40. A kit for simultaneously detecting in a sample the presence of one or more of a plurality of different target nucleic acids comprising (a) a plurality of nucleic acid primers wherein (i) for each target nucleic acid at least one predetermined primer is used which is specific for that target nucleic acid, (ii) each primer has a mass tag of predetermined size bound thereto via a labile bond, and (iii) the mass tag bound to any primer specific for one target nucleic acid has a different mass than the mass tag bound to any primer specific for any other target nucleic acid, and (b) instructions for use.
41. A kit for simultaneously detecting in a sample the presence of one or more of a plurality of different target nucleic acids comprising (a) a plurality of nucleic acid primers wherein (i) for each target nucleic acid at least one predetermined primer is used which is specific for that target nucleic acid, (ii) each primer has a mass tag of predetermined size bound thereto via a labile bond, and (iii) the mass tag bound to any primer specific for one target nucleic acid has a different mass than the mass tag bound to any primer specific for any other target nucleic acid; (b) a mass spectrometer; and (c) instructions for simultaneously detecting in a sample the presence of one or more of a plurality of

different target nucleic acids using the primers and the mass spectrometer.

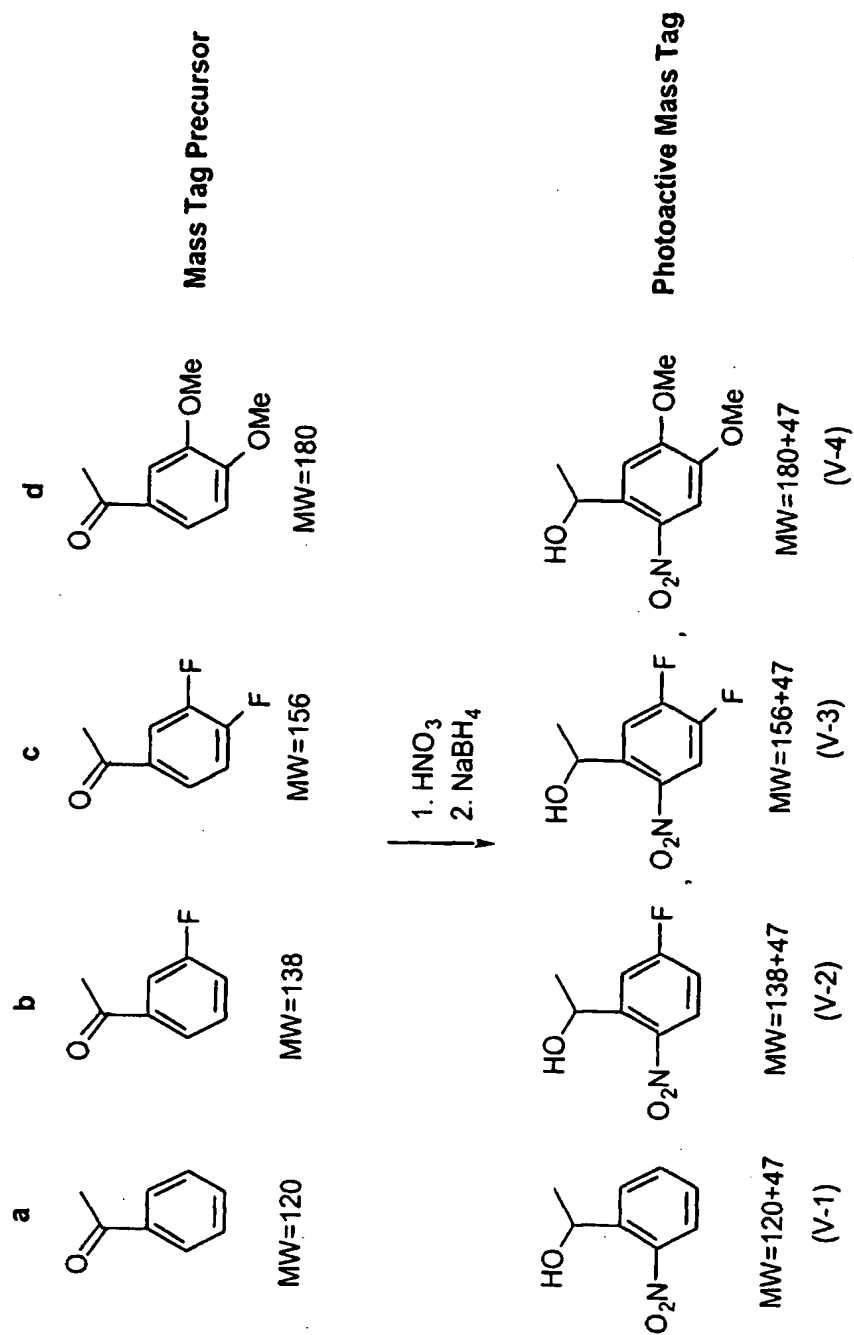


FIG. 1

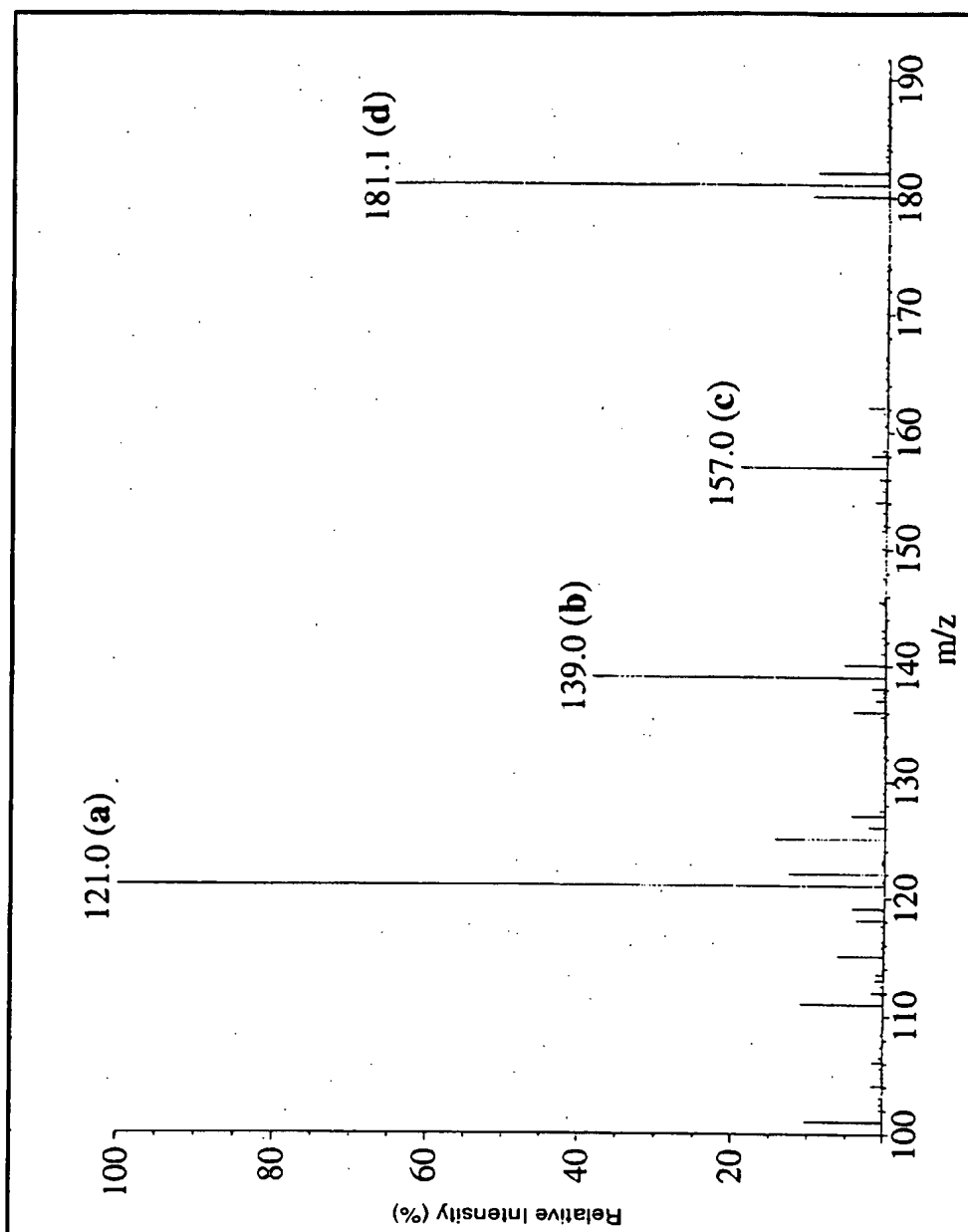
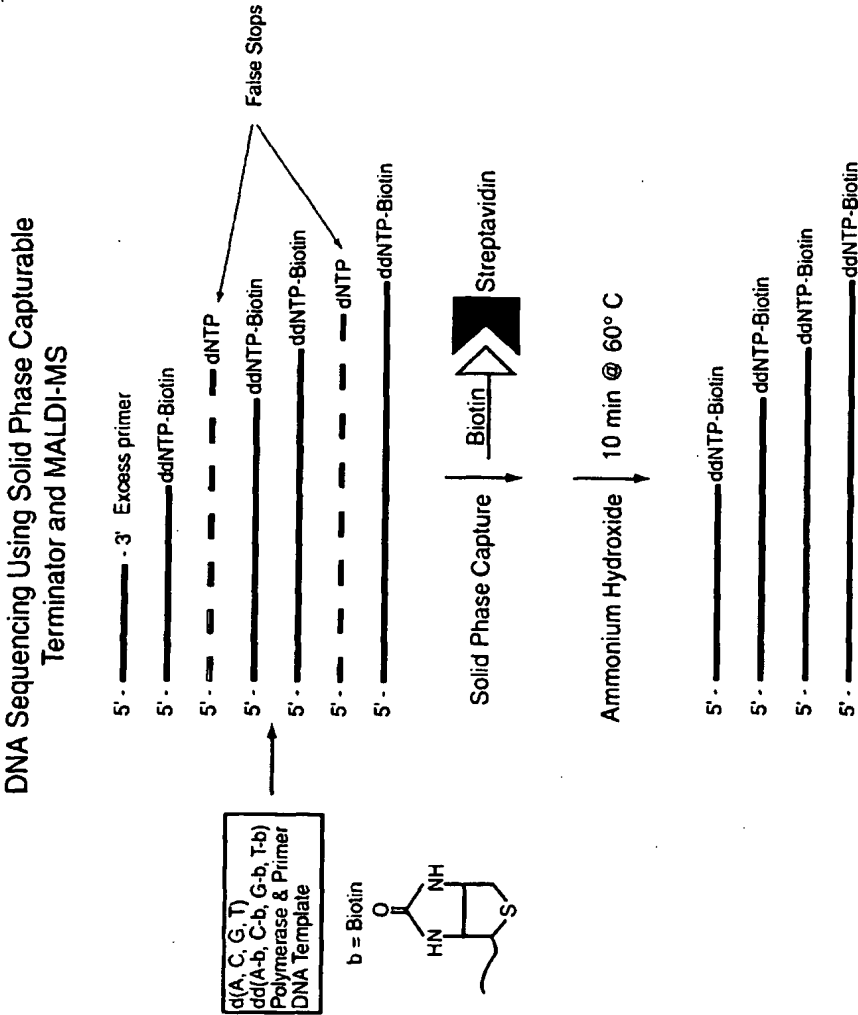


FIG. 2



One Tube Reaction; No Labels Required; Accurate Sequencing Data

FIG. 3

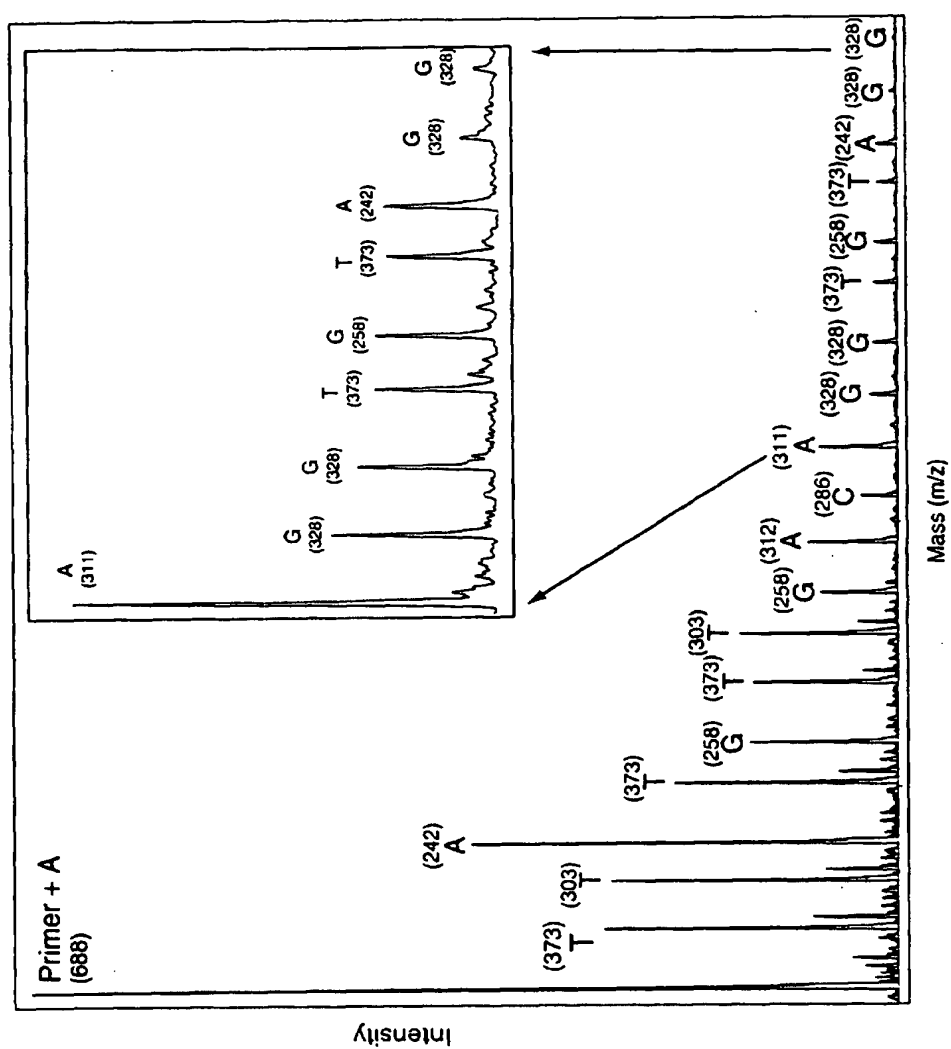


FIG. 4

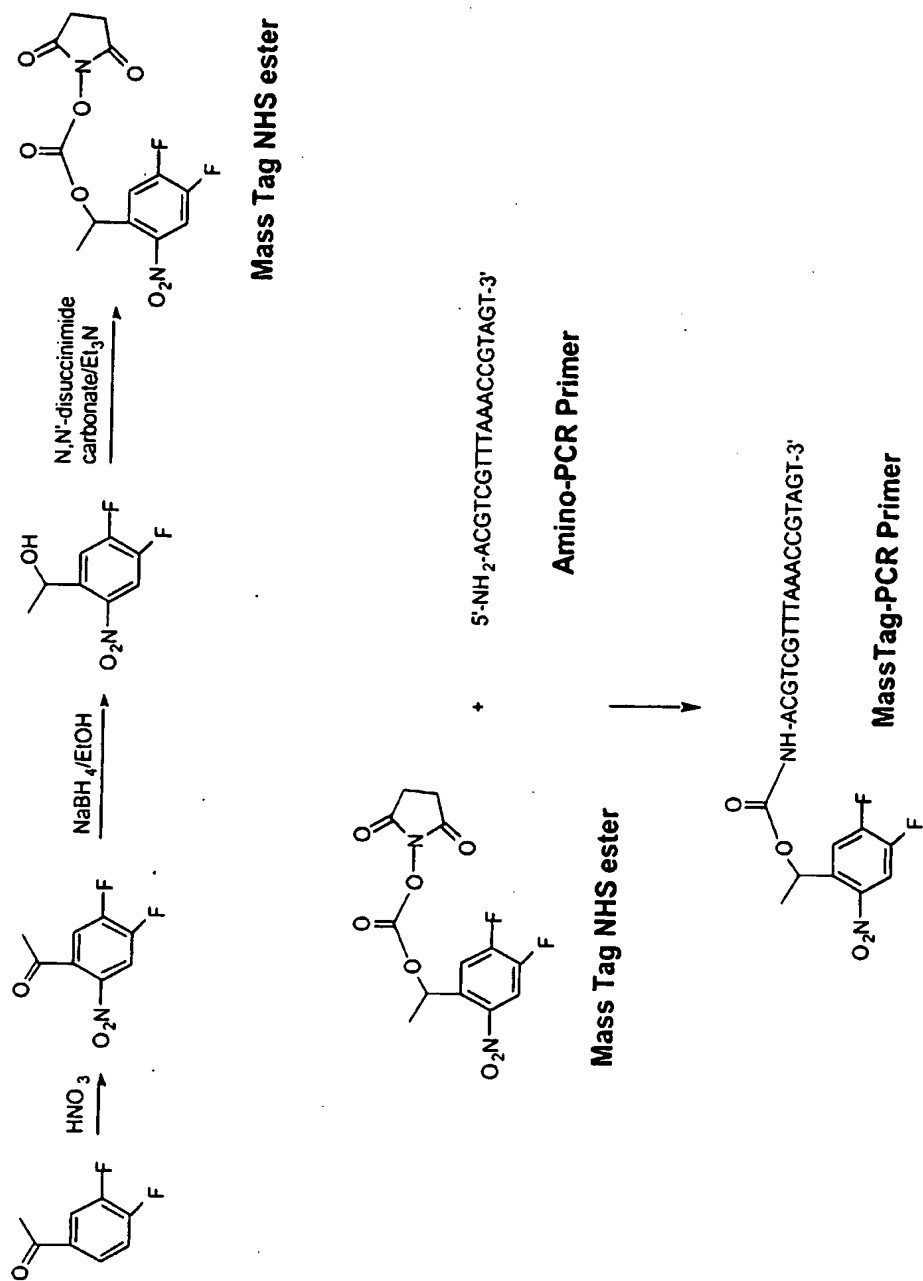


FIG. 5

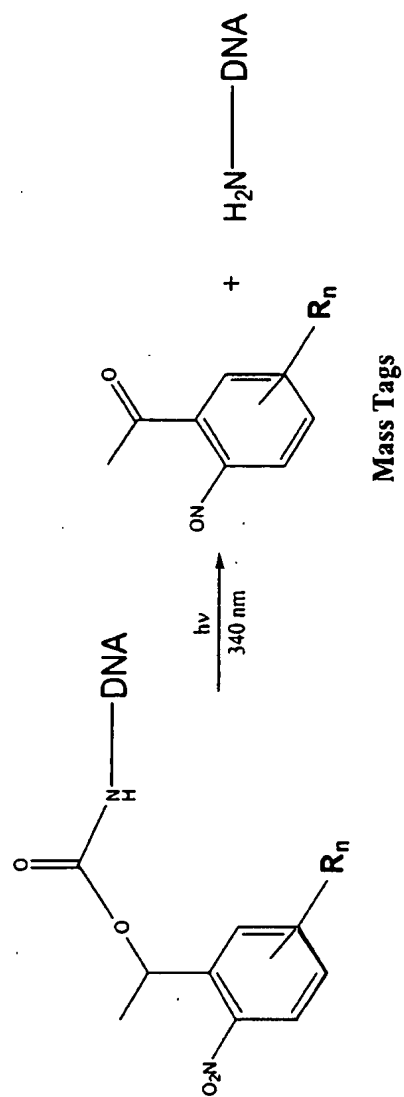


FIG. 6

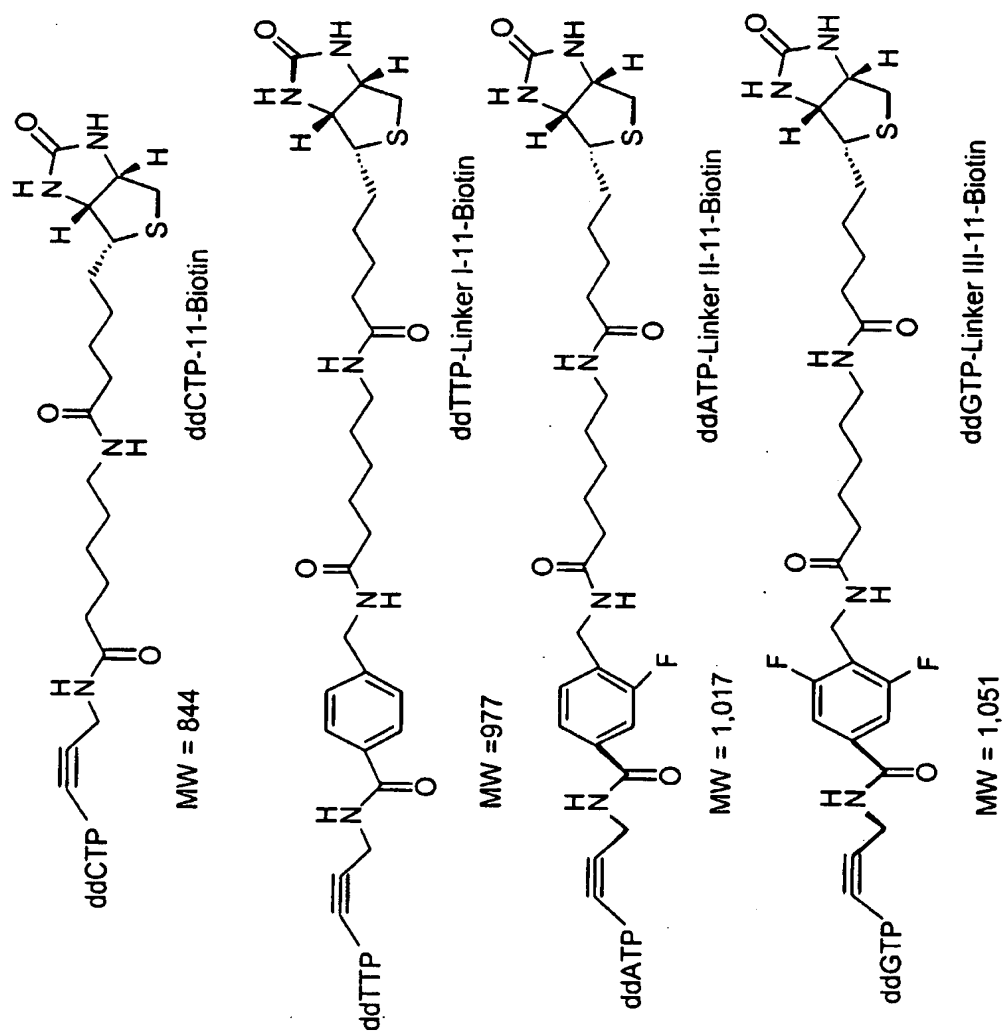


FIG. 7

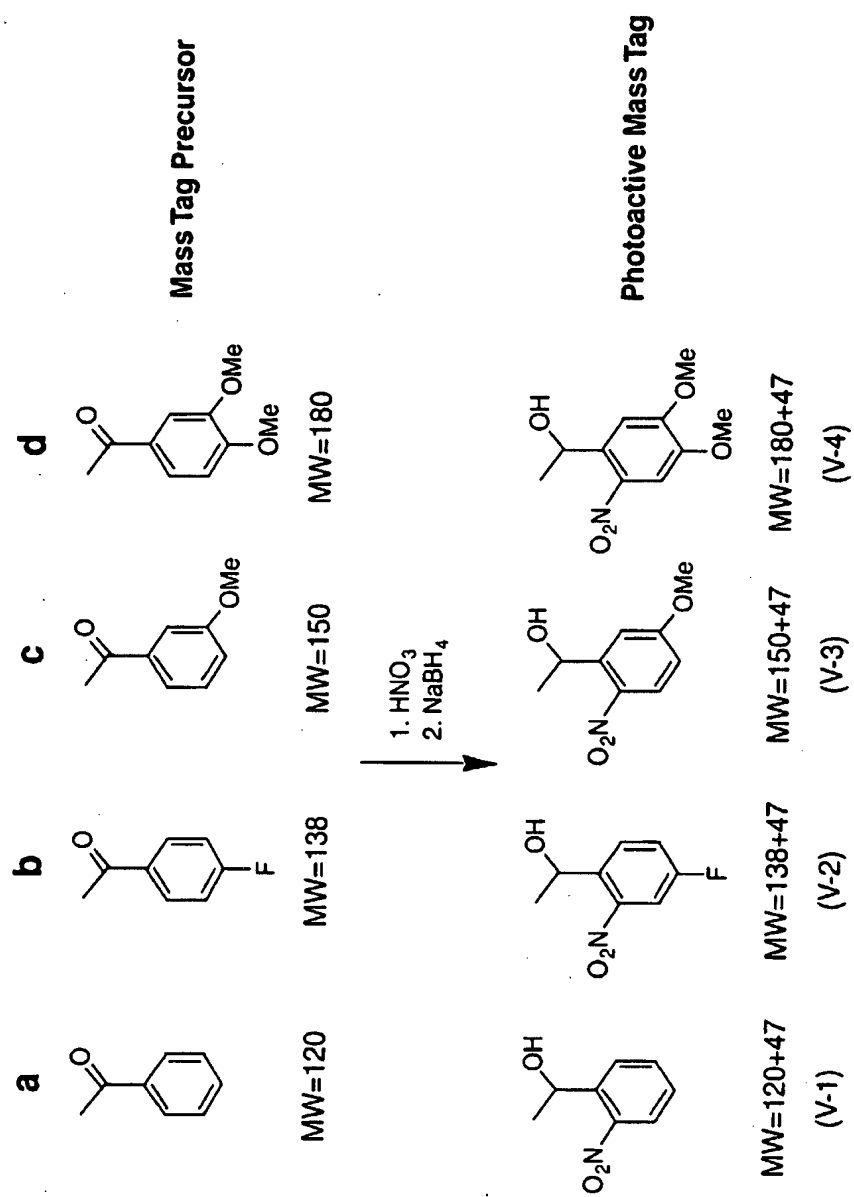


FIG. 8

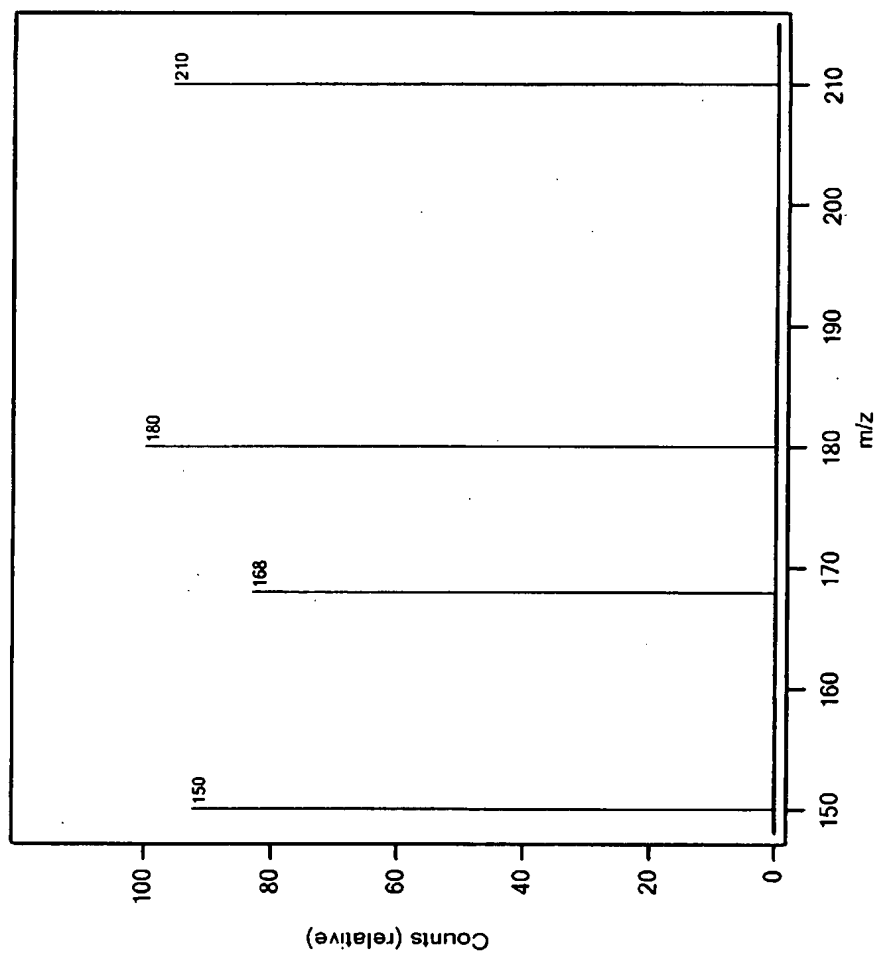


FIG. 9

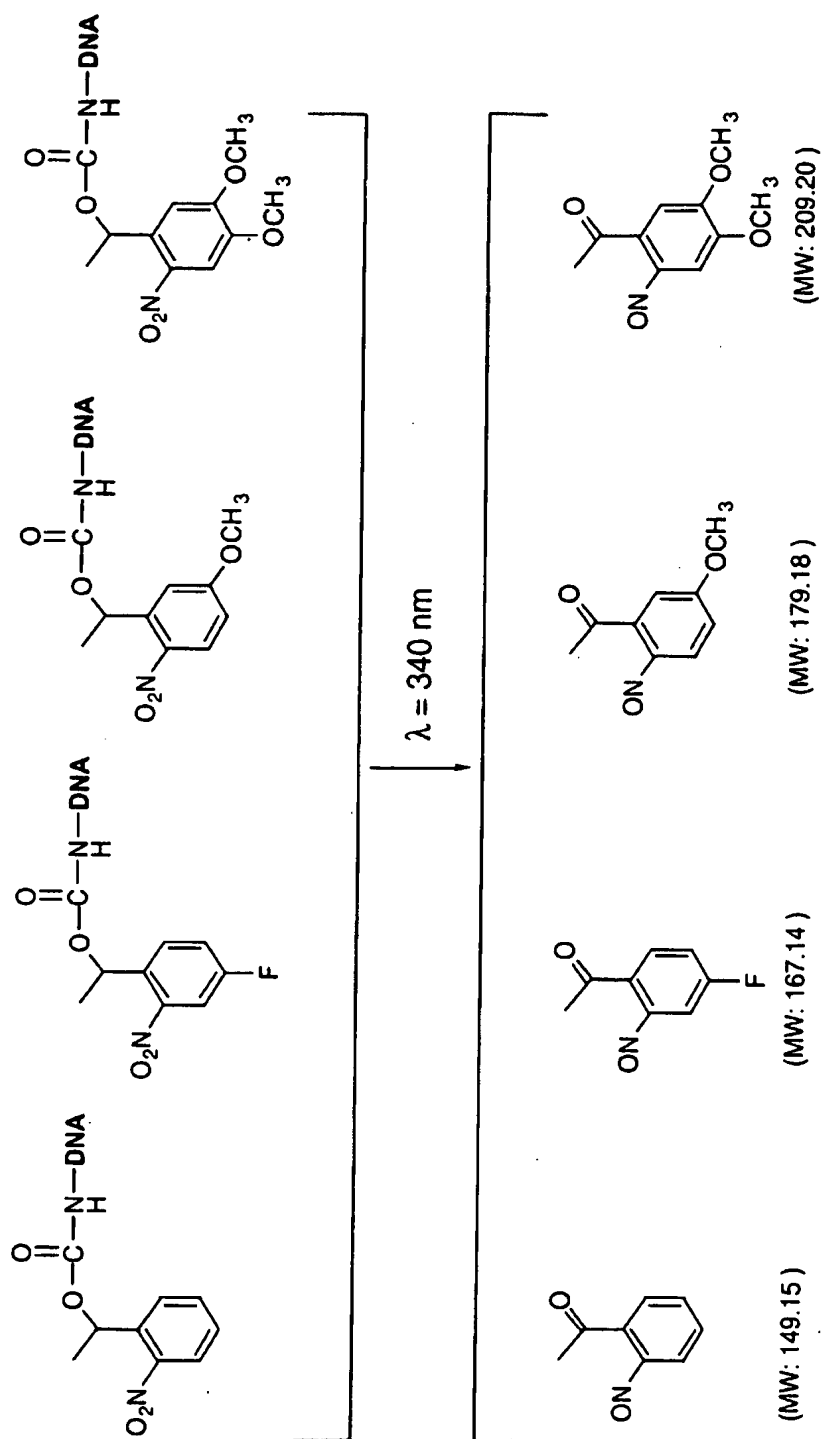


FIG. 10

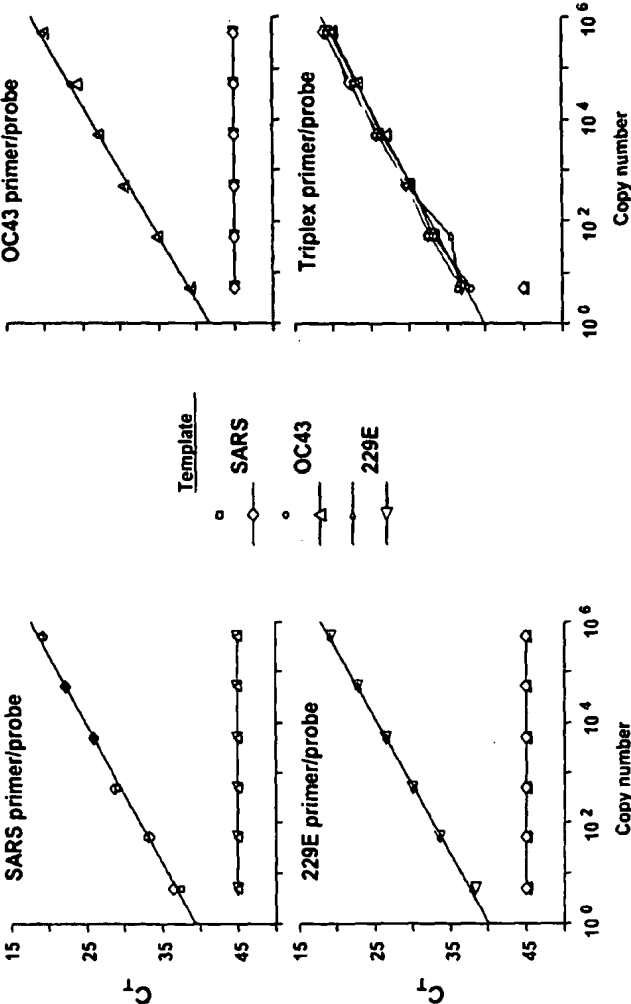


FIG 11.

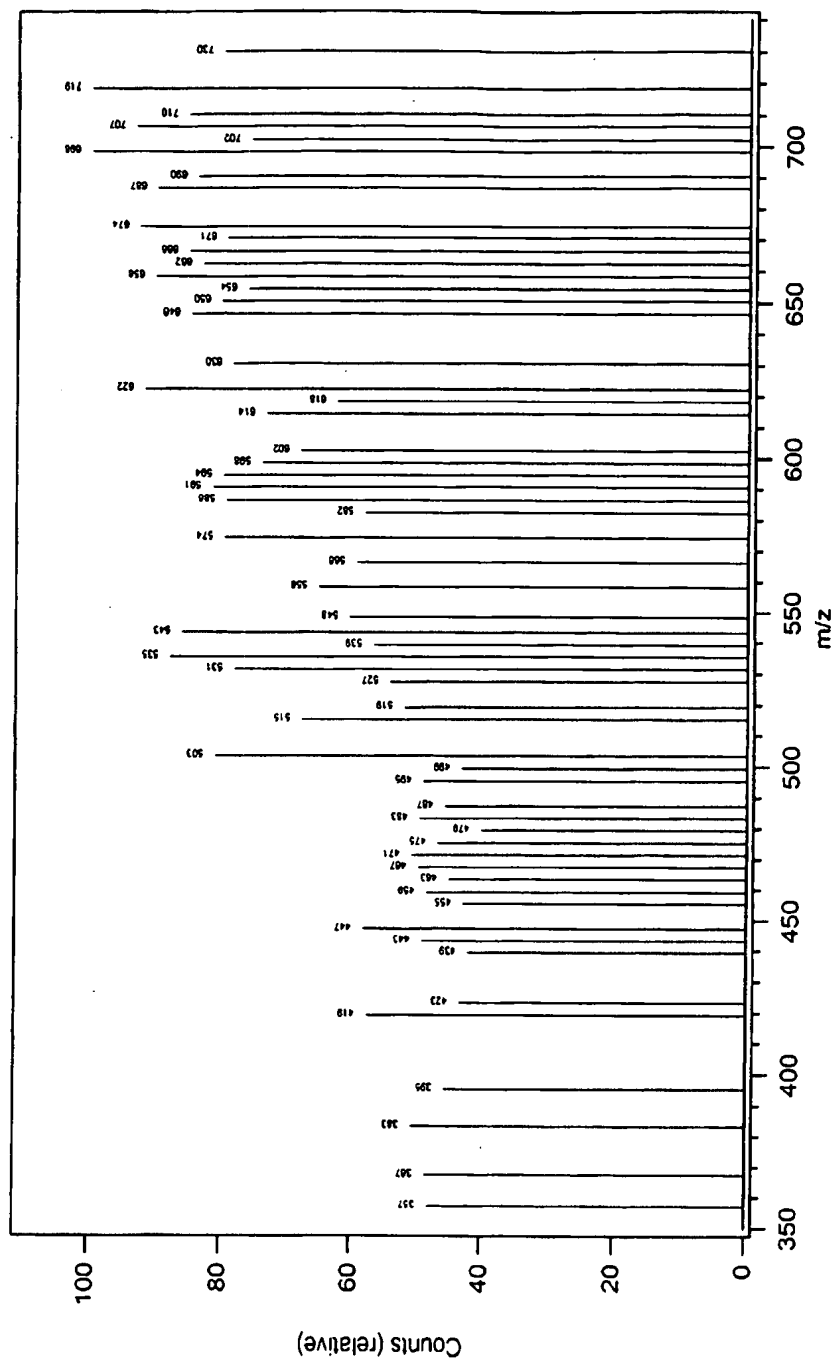


FIG. 12

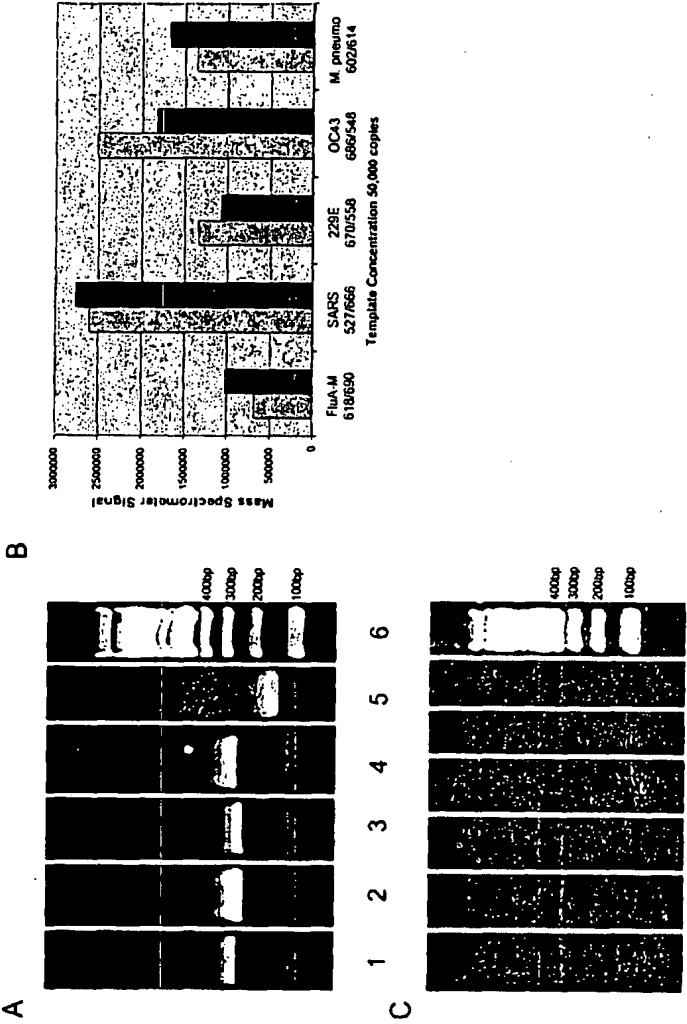


FIG. 13

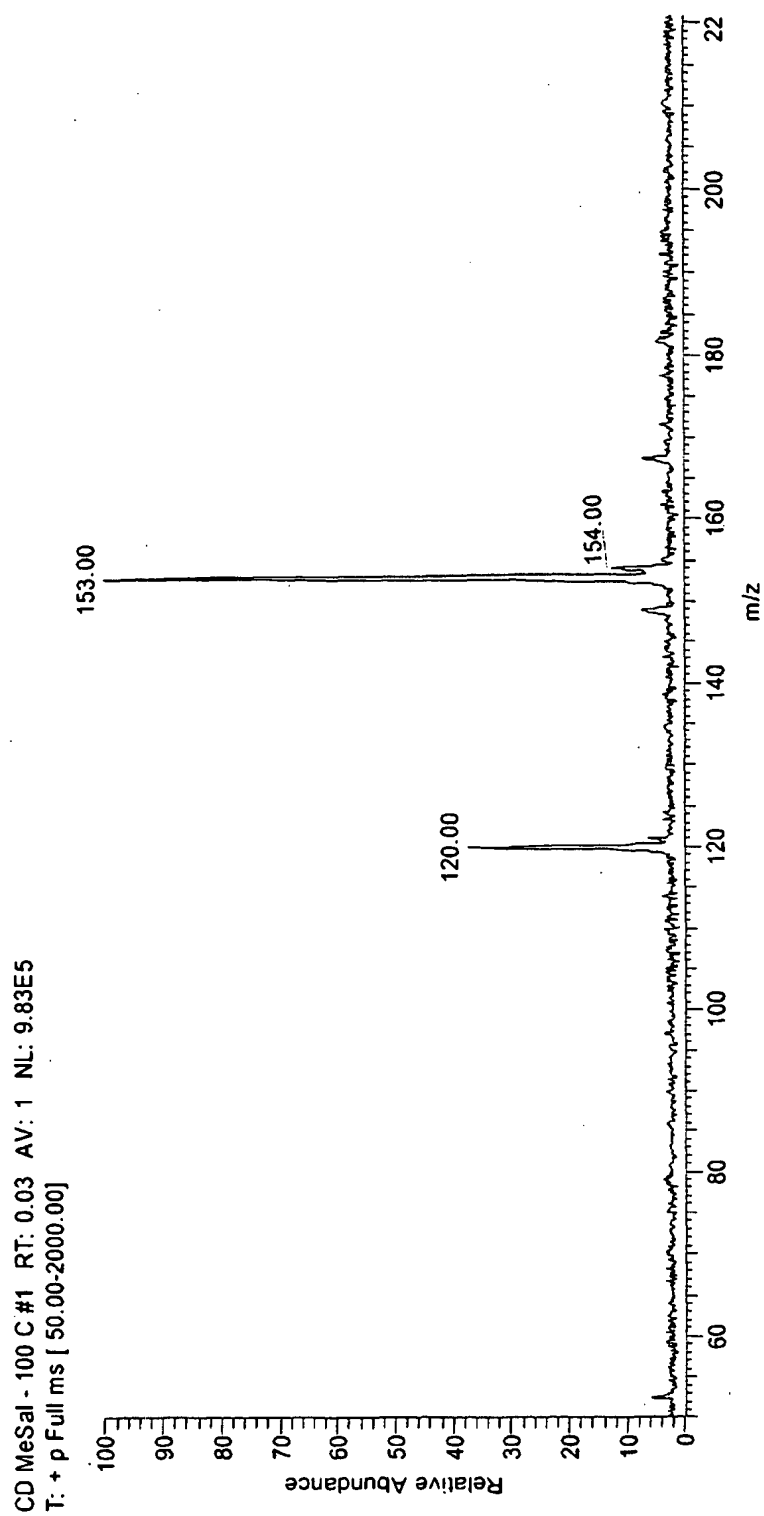


FIG. 14

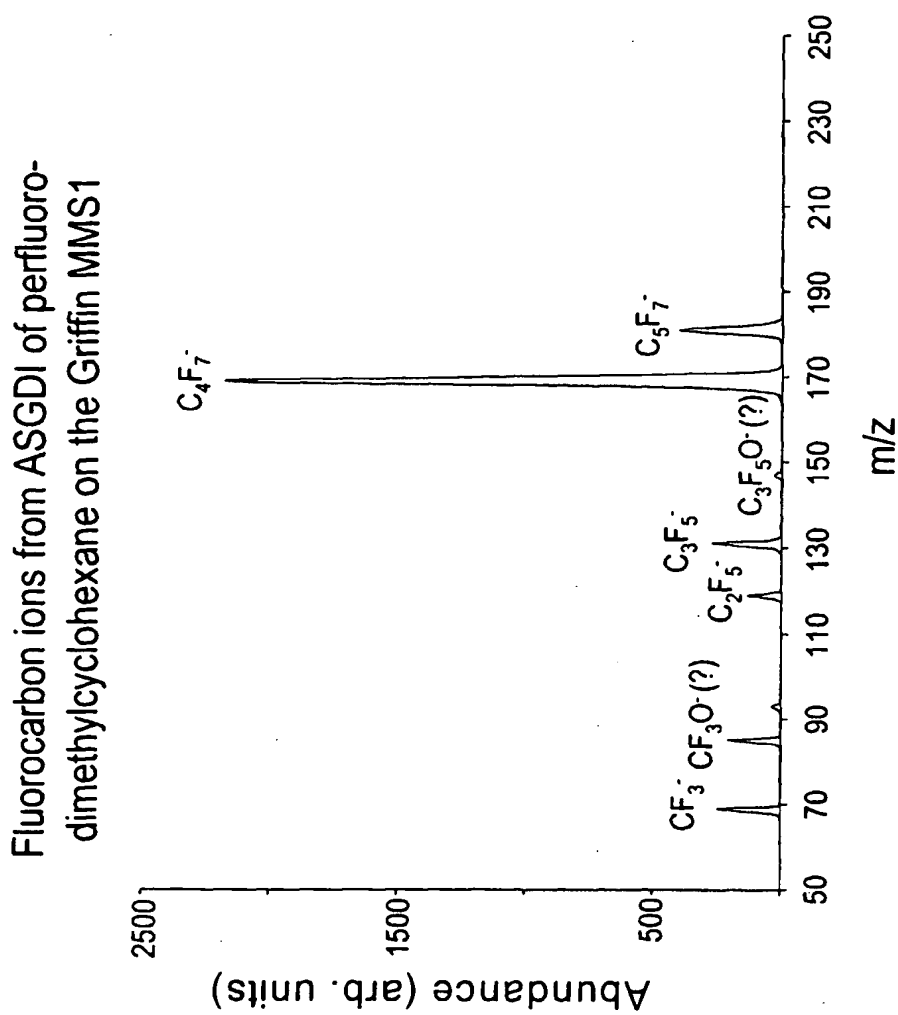


FIG. 15

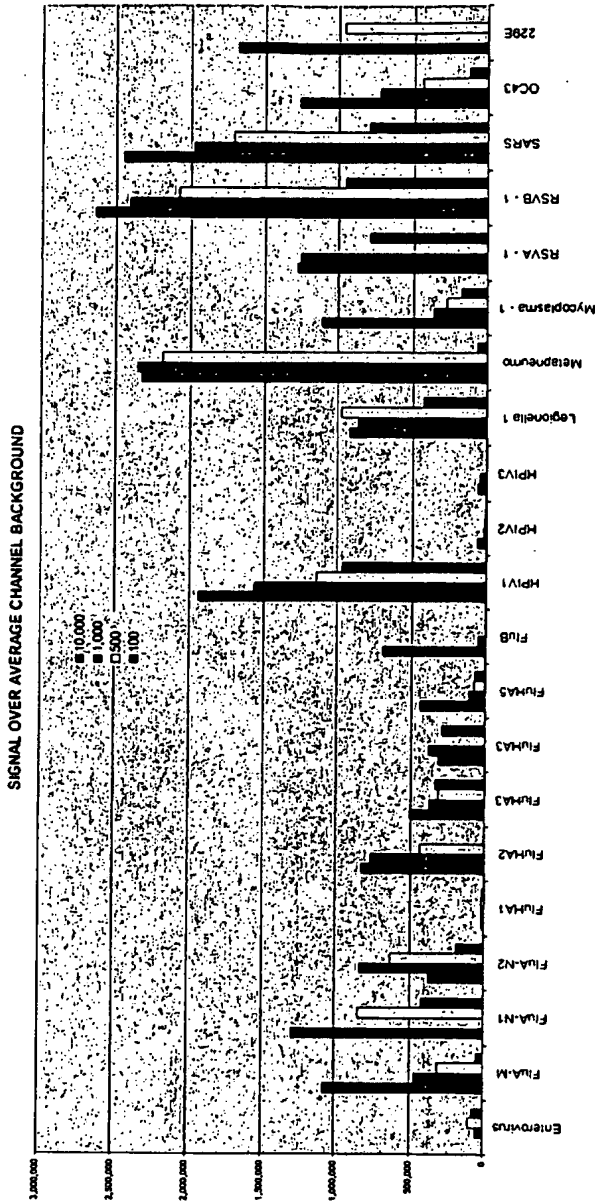


FIG. 16

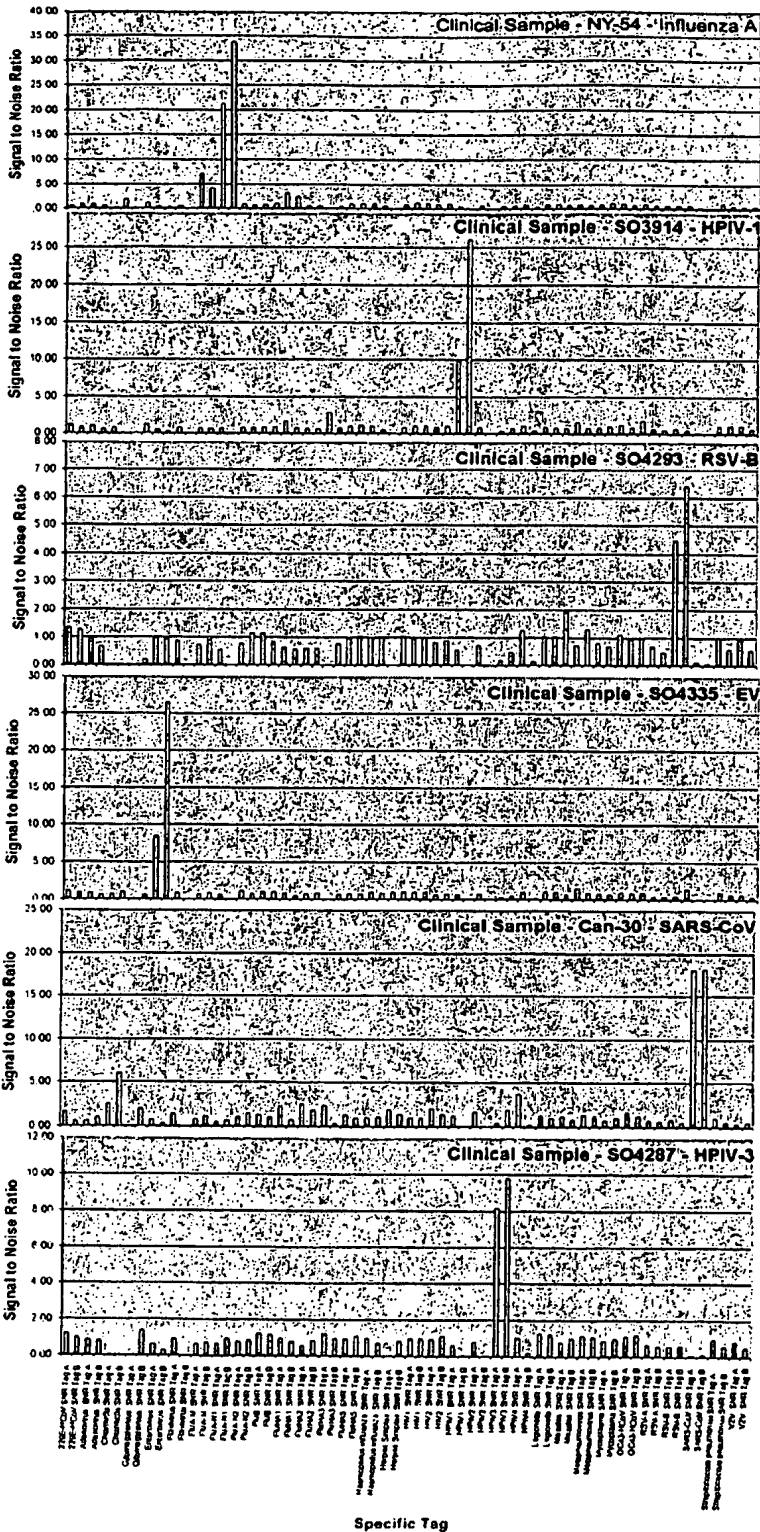
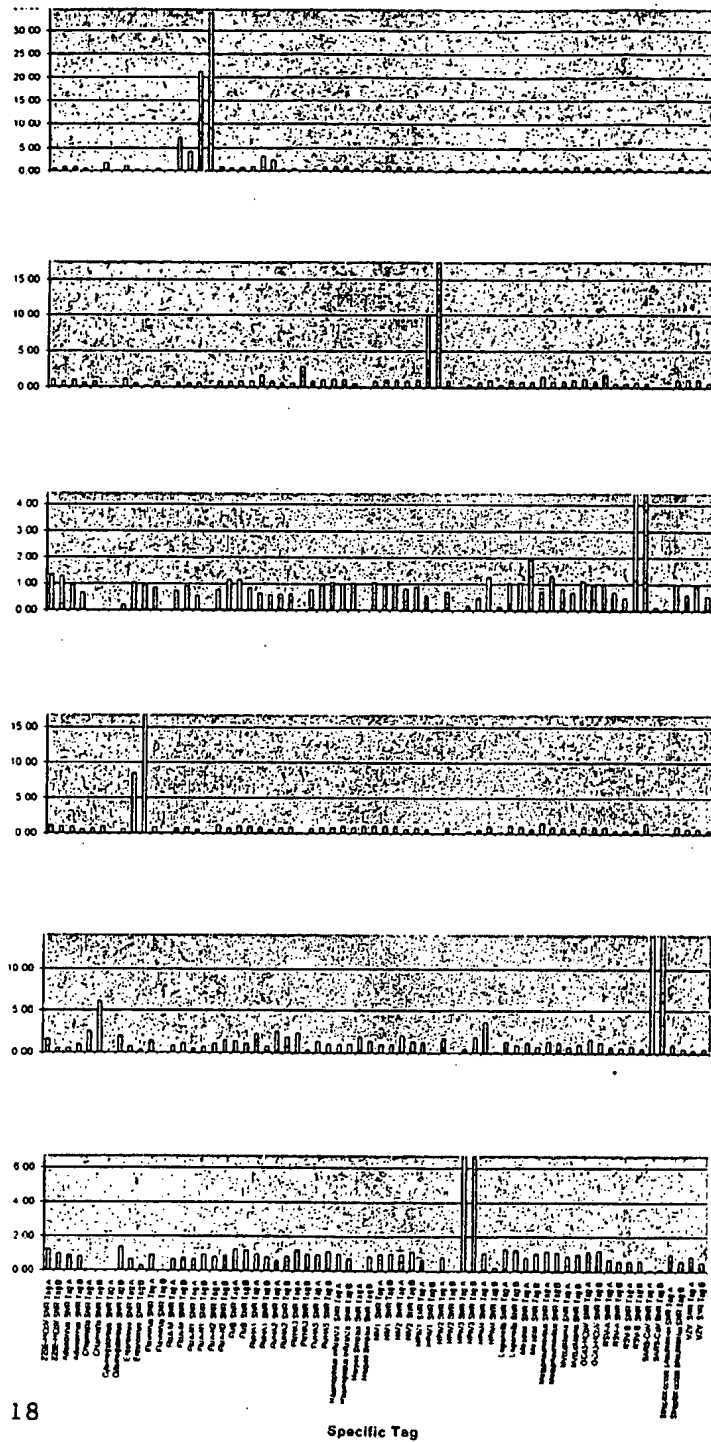


FIG. 17



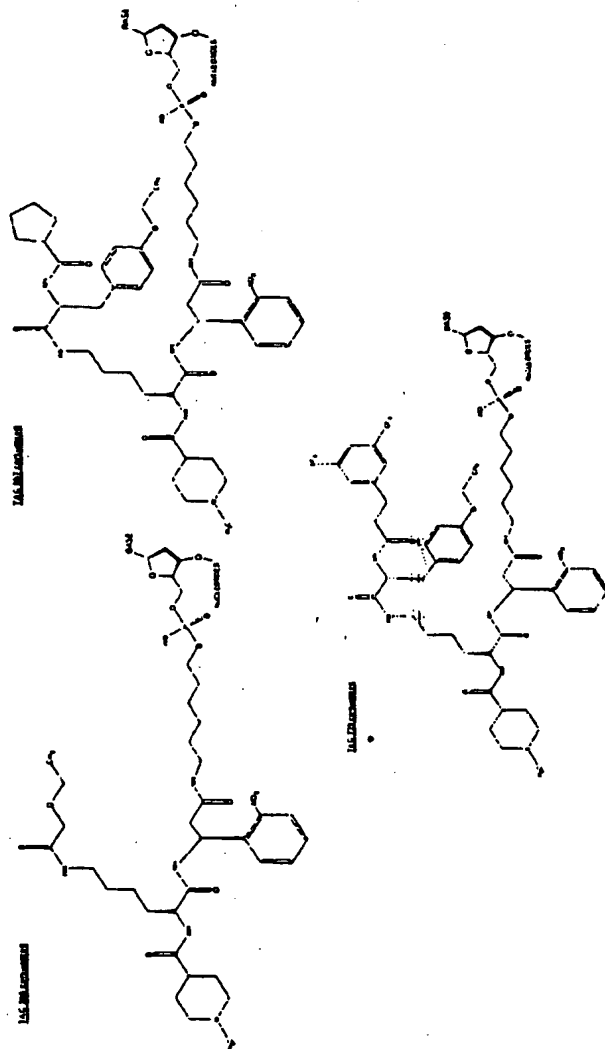


FIG. 19

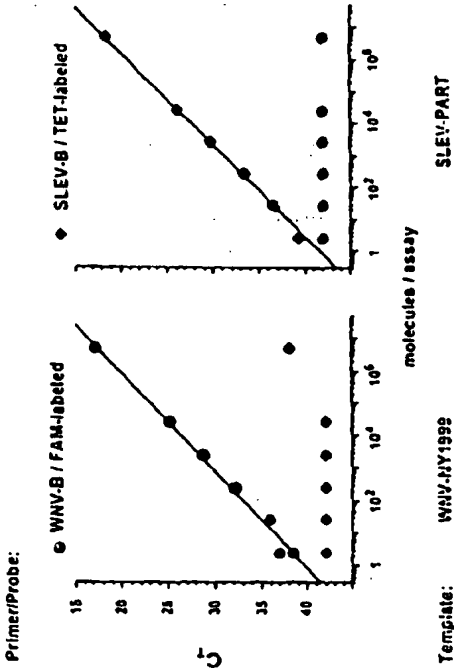


FIG. 20

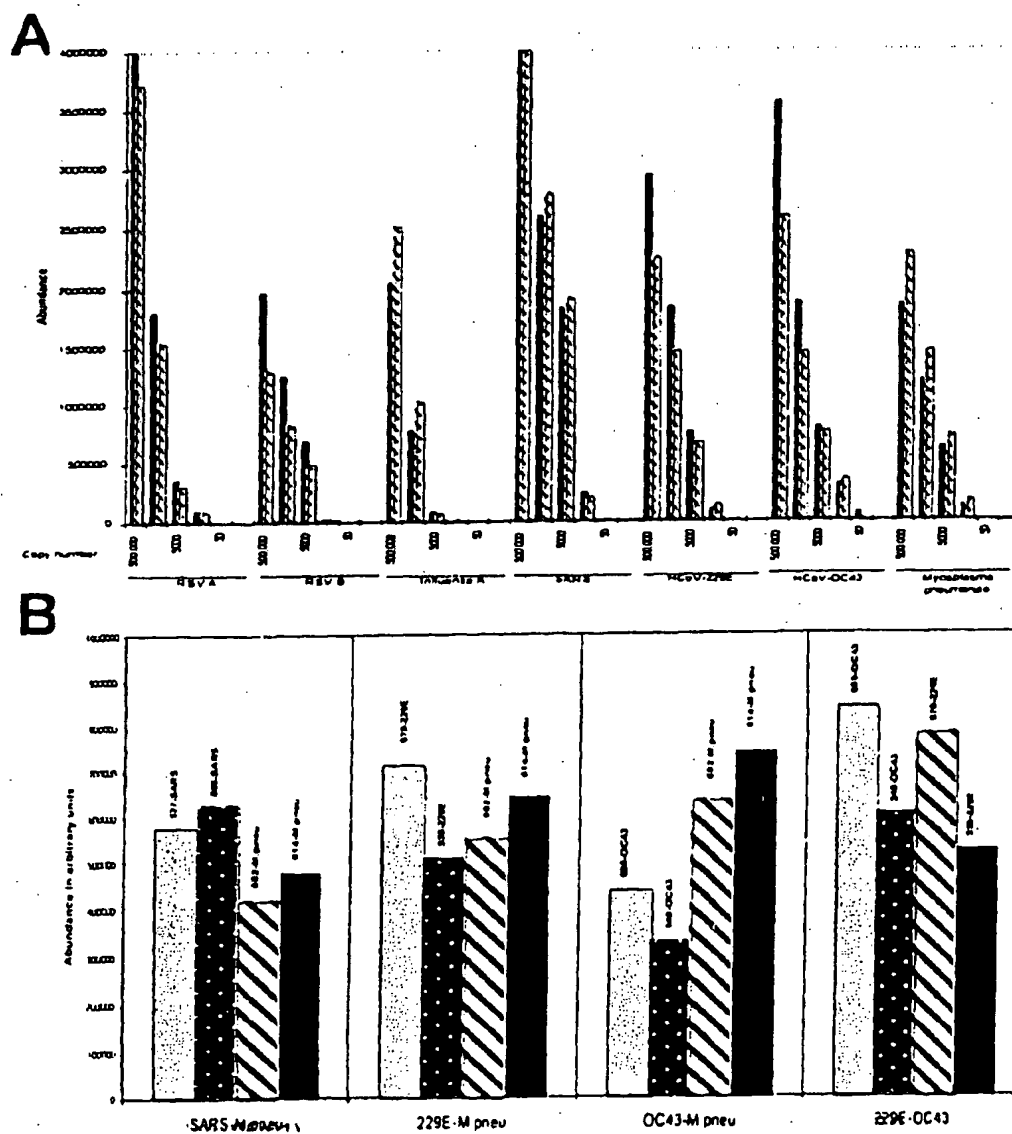
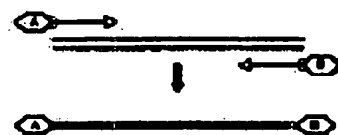
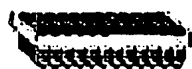


FIG. 21

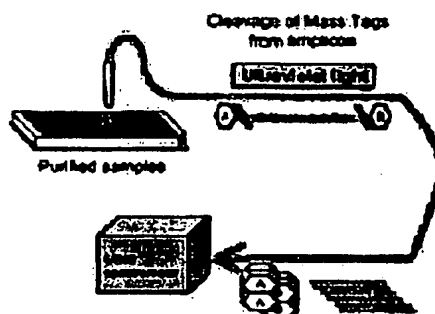
1. PCR amplification with Mass Tag primers 4. Automated sample injection, photocleavage



2. Product purification on filter plate



3. Elution into 96-well loading plate for mass spectrometer analysis



5. Detection and pathogen identification

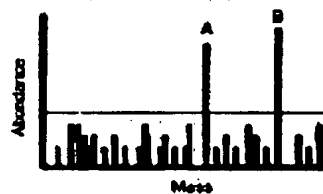


FIG. 22

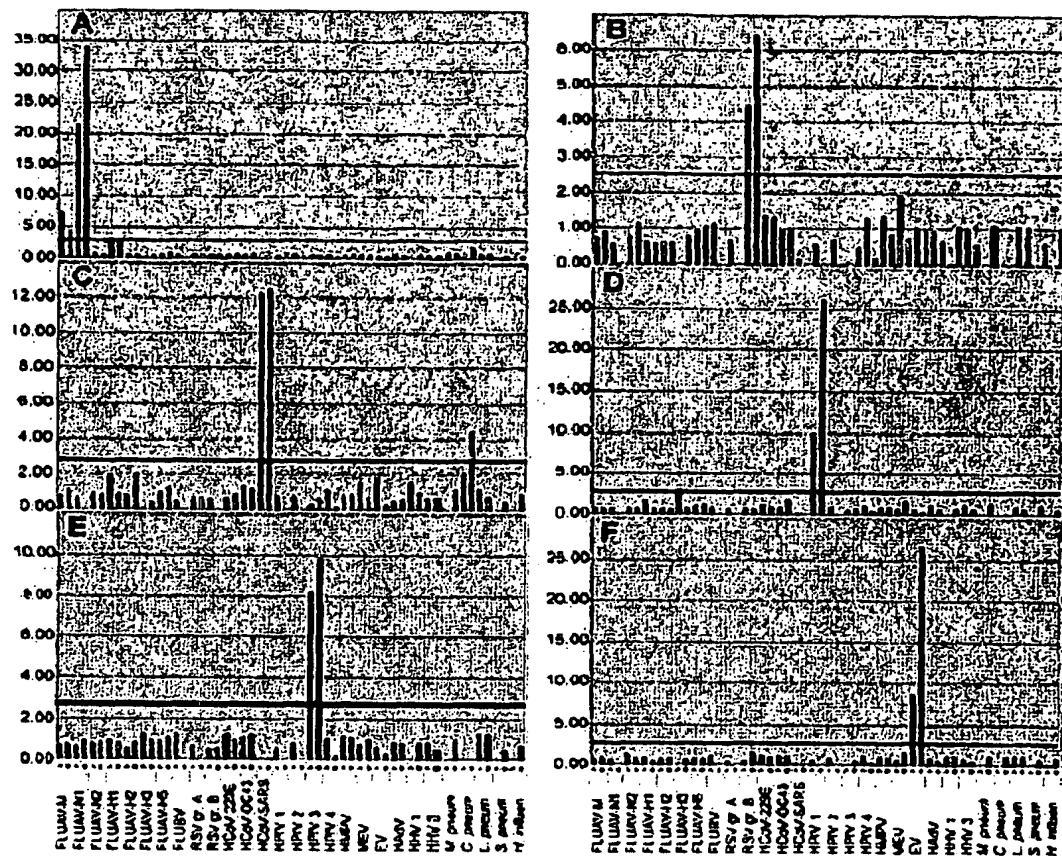


FIG. 23

SEQUENCE LISTING

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Jingyue, Ju
Thomas, Brieese

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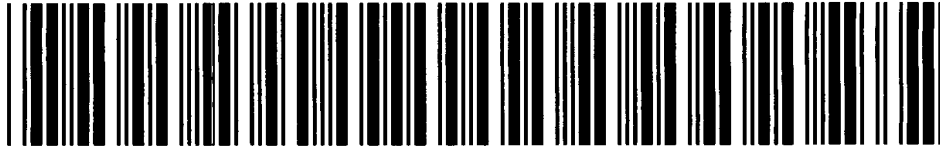
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Attachment A

- 1) Page 1; lines 5-7
- 2) Page 12; lines 9-10
- 3) Page 13; line 4
- 4) Page 17; line 3 to Page 22; line 4
- 5) Page 83; lines 7-8
- 6) Pages 96-112
- 7) Page 119; claim 38 to Page 121; claim 41
- 8) Page 122
- 9) Figure 23

IDS REFERENCES



☐ FOR